# MEMBRANE PROTEINS OF ALCELAPHINE HERPESVIRUS 1

By

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Alcelaphine herpesvirus 1 (AHV-1) is carried as a latent inapparent infection in Alcelaphine bovid species. In contrast, when cattle and several species of exotic bovids are infected, mortality approaches 100%. Although AHV-1 is endemic to and is largely confined to Africa, a very similar disease thought to be carried by sheep is found in the United States. Both diseases are known as malignant catarrhal fever (MCF). Our overall goal was to study the membrane proteins of AHV-1 and identify those molecules that play a role in the initiation of virus infection. We intended to apply this information to the development of novel methods of diagnosis of latently infected carrier animals. The antigenic relationships of AHV-1 and other herpesviruses infecting cattle were also examined, in the hopes of identifying a protein entirely unique to AHV-1 that might be used as a reagent in the development of a diagnostic test. We were able to identify a minimum of 6 putative virus-induced membrane proteins in AHV-1 infected cells, and produce monoclonal antibodies reactive with 4 of them. We were unable to identify a single protein as unique to

AHV-1, but determined that two monoclonal antibodies, 12B5 and 31B3, reacted with epitopes unique to AHV-1. Monoclonal antibody 12B5 exhibited neutralizing activity and was used to assemble a competitive enzyme-linked immunosorbant assay. The assay tested the ability of serum known to contain antibodies against AHV-1 to compete with the monoclonal antibody 12B5 for binding to AHV-1 infected cell antigen. Preliminary results indicated that an assay of this type represents a feasible method of diagnosis. The test also detected antibodies in sheep that were capable of competing with the monoclonal antibody, supporting the concept that the African form and the sheep associated form of MCF are caused by related viruses. Since the monoclonal antibody 12B5 possessed neutralizing activity, the proteins bound by this protein were examined more closely. The antibody immunoprecipitated a complex of 5 proteins whose precursor/product relationships were elucidated. The mature form of the protein found in the virion was identified as a disulfide-linked dimer.

# CHAPTER 1

# INTRODUCTION

## Historical Perspective

Alcelaphine herpesvirus 1 (AHV-1) is carried as a latent infection by African antelope of the family Bovidae, subfamily Alcelaphine. Alcelaphine species include wildebeest (Connochaetes) hartebeest (Alcelaphus), and topi (Damaliscus), and are not clinically affected by infection. In contrast, infection in cattle, exotic bovids, and several species of deer results in a generalized lymphoproliferative disease with a mortality approaching 100% (18,46,48).

The disease in susceptible species has been known as malignant catarrh, malignant head catarrh, snotsiekte, and malignant catarrhal fever (MCF). Malignant catarrhal fever due to Alcelaphine herpesvirus 1 is an exotic disease but is important to the United States for several reasons. First, MCF has been diagnosed on several occasions in the United States since the 1970s, and many speculate that the incidence of MCF is presently increasing (20,35). Sporadic outbreaks pose an expanding threat to the domestic cattle population as well as susceptible wildlife species. Second, increased popularity of exotic game ranching in areas such as Texas has provided increased opportunities for the dissemination of the disease in nearby cattle operations. Third, zoological collections have suffered substantial

mortality in endangered species as a result of transmission of AHV-1 (9,16).

Epidemiologic data suggest that sheep may also serve as reservoirs for MCF. The sheep-associated form of MCF was first described in Europe in 1798 and has been called the European or American form, although its distribution is worldwide (21). This endemic disease is clinically and pathologically indistinguishable from the disease caused by AHV-1. Serological surveys have indicated that approximately 27% of sheep and goats possess antibodies reactive with AHV-1 by virus neutralization, immunofluorescence, and ELISA (22). As early as 1929 investigators were successful in transmitting the sheep-associated form by inoculation of infected blood, although no virus has yet been isolated from sheep or clinically infected animals associated with sheep (53,54).

The wildebeest-associated or African form of MCF has been known to the Masai tribesmen of eastern Africa for centuries, and it has been estimated that 7% of the annual mortality in cattle in eastern Africa is due to this disease (40). Masai pastoralists have long realized the importance of preventing the exposure of their cattle to the wildebeest, especially during the wildebeest calving season. This association of wildebeest and MCF was first investigated in 1923 when Mettan raised a wildebeest calf on a Friesen cow, which resulted in the death of all exposed cattle. The etiological agent, AHV-1, was first isolated in 1960 from a blue wildebeest (46).

## Clinical and Pathological Manifestations

MCF is an acute, generalized disease with mortality near 100%. It is characterized by fever, depression, profuse nasal and ocular discharge and encrustation, drooling, photophobia, keratitis, erosion and diptheresis of mucous membranes, generalized lymphadenopathy, skin lesions, and, occasionally, cystitis and central nervous involvement (20). Gross post mortem lesions vary considerably depending on the course of the disease. Animals that die very acutely may show fewer lesions other than hemorrhagic enterocolitis. In more protracted cases, lesions are more severe. The muzzle is often encrusted and raw, and cutaneous lesions may be hyperemic and occur as a generalized exanthema with exudation of lymph. These lesions are frequently seen on the ventral thorax and abdomen, inguinal region, perineum and loin. Lesions in the respiratory system may vary from mild serous nasal discharge and hyperemia of the mucous membranes to severe ulceration of epithelia and formation of prominent pseudomembranes. Epithelia of the nasal passages and sinuses may be affected as well as pharyngeal, and laryngeal epithelium. Lesions may also extend to the tracheal and bronchial mucosa. The mucosa of the alimentary tract may vary from mild mucosal hyperemia and edema to severe erosions and ulceration on the dental pad and gingival surfaces, tongue and buccal papilla. Mucosal inflammation, hemorrhage and ulceration may also be found in the rest of the gastrointestinal tract including the esophagus, abomasum, small intestine, colon and rectum. If lesions are present in the urinary tract, they usually include hyperemia of the bladder

mucosa and, occasionally, marked dilatation of bladder mucosal vessels. Petechiae and ecchymoses may also occur in the renal pelvis and ureters. The liver is usually slightly enlarged with a prominent reticular pattern. There may be hemorrhages and erosions in the gall bladder. A generalized lymphadenopathy is characteristic of MCF and is most consistently prominent near the head and neck. Fibrinous polyarthritis has been seen in a number of cases of MCF. Corneal edema, with ulceration and/or vascularization is often seen. Malignant catarrhal fever can be confused with other viral diseases, namely bovine viral diarrhea-mucosal disease, rinderpest, bluetongue, foot and mouth disease, and vesicular stomatitis. These diseases may all result in similar gross lesions which necessitate histopathologic and serologic examination for diagnosis (20).

Lesions that provide a consistent basis for diagnosis of MCF are found in the epithelia of the gastrointestinal and urinary tracts, the lymphoid tissue and the adventitia and walls of small blood vessels in any organ. Vascular lesions consist of fibrinoid necrotizing vasculitis with a predominantly mononuclear cell infiltrate of the adventitia. Lymphoid tissue shows destruction and loss of lymphocytes and an increase in macrophages and lymphoblast proliferation.

Several investigators have pointed out the similarity of lesions of lymphoproliferation with those of malignant lymphoma and infectious mononucleosis, raising speculation as to the oncogenic potential of AHV-1 (23,26). In addition, recent work in which AHV-1 was inoculated into rats describes neoplastic-like lymphoproliferation

in gut-associated lymphoid tissue. This lesion may represent a potential model for herpes virus-induced oncogenesis (24).

## Transmission

Alcelaphine species harbor the virus as a latent infection and are apparently unaffected clinically. The virus is thought to be highly cell-associated in the host and is rarely transmissible between adult wildebeest, although virus has been isolated from nasal secretions of captive adult wildebeest after stress or administration of corticosteroids (56).

Neonatal wildebeest have been shown to shed cell-free AHV-1 in nasal and ocular secretions and in feces (38). Virus can be isolated from infected wildebeest calves up to 3 months of age, which coincides with the appearance of neutralizing antibodies in the nasal secretions (41). Transmission to cattle or other susceptible species is thought to occur by inhalation of cell free virus from aerosol droplets or ingestion of food or water contaminated with infectious secretions or feces (40). The Masai of eastern Africa have firmly believed for centuries that transmission of MCF occurs from contact of cattle with fetal membranes and birth fluids from calving wildebeest. However, attempts to isolate virus from fresh placentas from calving wildebeest have been unsuccessful (55).

Virus is also apparently highly cell-associated in susceptible species, and cattle-to-cattle transmission has not been demonstrated to be significant (47). The mode of transmission of the sheep associated form of MCF is unknown, but is believed to require close

contact with carrier animals and does occur with greater incidence near the lambing season. Both wildebeest-associated and sheep-associated forms of malignant catarrhal fever have been experimentally transmitted to a variety of susceptible bovids and cervids, as well as rabbits, guinea pigs, rats and hamsters, by inoculation of infected blood and/or other tissues (8,25,27,31,42,45,63).

Two genetically distinct groups of AHV-1 have been identified, and are designated AHV-1 and AHV-2. AHV-1 refers to virus isolated from wildebeest, and AHV-2 refers to virus isolated from topi and hartebeest (30). Epidemiological data suggests AHV-2 has not been associated with MCF in cattle (50). AHV-1 and AHV-2 have been demonstrated to be in two distinct groups by restriction endonuclease patterns and DNA cross-hybridization (57).

## Virus Characteristics

Alcelaphine herpesvirus 1 has been examined by electron microscopy of infected cell monolayers. The virions have typical herpesvirus morphology and have icosohedral nucleocapsids of 94-194 nm. They apparently develop in the nucleus and pass through the nuclear membrane, acquiring an envelope (7).

The best source of virus for isolation is buffy coat cells which are then cocultivated with fibroblasts. Although less consistent, virus isolation can also be made from spleen, lung, lymph nodes, adrenals and thyroids. Virus is quickly inactivated in the carcass, and tissues must be collected within one hour post-mortem for virus isolation to be successful.

Cytopathic effect (CPE) may not be evident before several subculture passages of cells. Early CPE consists of formation of syncytia followed by contracting and rounding of cells. As the virus becomes more adapted to cell culture, there is formation of focal clusters of shrunken rounded cells, and a decrease in the formation of syncytia. Cowdry type A inclusion bodies are formed in infected cell cultures, but have not been identified in tissues of AHV-1-infected animals (20).

The virus apparently infects lymphocytes, and cocultivation of lymphocytes with fibroblast feeder layers results in infection of the fibroblast cell lines. Because of this ability to infect lymphocytes, AHV-1 has been classified as a gamma herpesvirus, the same designation given to Epstein-Barr virus, Marek's disease virus, Herpesvirus saimiri and Herpesvirus ateles. The first wildebeest virus, isolated by Plowright in east Africa in 1960, is designated WC11 and has become the prototype laboratory strain. This virus has been adapted to grow in variety of cells including bovine, ovine, rabbit and African Green Monkey kidney (Vero) cells. At 37°C AHV-1 forms syncytia in susceptible cell lines, which slowly round up and detach (15). If grown at lower temperatures of 32-34°C, development of CPE is slightly accelerated and larger amounts of cell-free virus are produced (15). The replicative cycle of WC11 is quite long, and foci of CPE are not visible until 72-96 hours post infection. A new AHV-1 isolate, designated 1982, was recently obtained from the Zoological Society of San Diego from a diseased Formosan sika deer (Cervus nippon) and has

been used in our laboratory for a portion of these studies. This isolate has proven very useful, as its replication cycle is considerably shorter than that of WC11. Extensive CPE resulting in complete destruction of the monolayer occurs in less than 72 hours with 1982, as opposed to 2-3 weeks with WC11. Virus titers are 2 to 3 logs higher with 1982 than with WC11.

Alcelaphine herpesvirus 1 has also been successfully isolated from hartebeest, topi, and the scimitar-horned oryx (Oryx gazella dammah), in addition to the species that are susceptible to induction of clinical disease by the virus. Analysis of DNA from these various isolates has demonstrated that all isolates except the hartebeest and topi isolates have a high degree of homology with the prototype WC11 strain of AHV-1. The DNA from the hartebeest and topi isolates hybridize with each other, but not with the isolates from other species (57).

In addition to Alcelaphine species, a high prevalence of neutralizing antibody to AHV-1 has been found in the subfamily Hippotraginae (addax and various oryx species), but no virus has yet been isolated from these species (37).

The DNA structure of AHV-1 appears very similar to that of H.saimiri and H.ateles, which are both gammaherpesviruses. DNA from purified virions sediments into two components in cesium chloride gradients which appear equivalent to the M and H components of saimiri and ateles. The M component is thought to consist of unique coding regions surrounded by flanking repeated sequences of varying length.

These are extremely high in guanine plus cytosine content relative to the non-reiterated DNA. The H component is thought to be a heterogeneous mixture of similar reiterated sequences (6).

The pathogenesis of the disease is poorly understood. Viral antigens have been demonstrated in mature lymphocytes by immunofluorescence, although only a small number of infected cells were detectable by this technique (44). Antigen has only rarely demonstrated in cells other than lymphocytes (52).

## Diagnosis

The similarity of the clinical and gross pathological signs associated with MCF and those of other viral diseases infecting cattle necessitates the use of serological means for definitive diagnosis. Serological identification of infected animals is, however, complicated by immunological cross-reactivity that exists among bovid herpesviruses. The assay most commonly used to test animals for latent herpes infections is an indirect immunofluorescence assay using virus-infected target cells. Unfortunately, this assay fails to discriminate animals carrying AHV-1 from those previously infected with other more common or less potentially devastating herpesviruses (19).

There are at least five herpesviruses infecting cattle, bovine herpesvirus 1 (BHV-1), bovine herpesvirus 2 (BHV 2), bovine herpesvirus 4 (BHV 4) pseudorabies virus (PRV or Suid herpesvirus-1), and alcelaphine herpesvirus 1.

Bovine herpesvirus 1 (BHV-1), also known as infectious bovine rhinotracheitis virus, is a very common disease of cattle which is associated with fever and mild to severe respiratory tract infections. These infections may be complicated by secondary infections with a variety of other viruses and bacteria. Encephalitis, abortions and occasional enteritis can result from BHV-1 infections. Because BHV-1 is so commonly encountered in cattle populations, the possibility of diagnostic cross-reactivity is a valid concern.

Bovine herpesvirus-2 (BHV-2), which is also known as bovine herpes mammillitis virus, is far less significant clinically, but may be responsible for occasional outbreaks of mammillitis and secondary bacterial mastitis. Epidemiological surveys estimate that 20% of the U.S. cattle population may be seropositive for BHV-1 (58). Previous work in our laboratory has demonstrated significant cross-reactivity between BHV-2, BHV-1, PRV and Herpes simplex virus type 1 (HSV-1). It therefore seems probable that there will be some cross-reactivity with this rather common virus and AHV-1.

Bovine herpesvirus 4 (BHV-4) encompasses a group of viruses of minor clinical significance which have been isolated from animals showing variable clinical signs. This group of viruses has also been referred to as bovine herpesvirus 3, a designation previously reserved for AHV-1. This group includes Movar virus, which we are using as a group prototype, as well as DN599, 66-P-347, and UT. Viruses in this group are closely related serologically, and restriction endonuclease patterns of DNA from various isolates appear quite similar (43). There is evidence that, of all the bovid herpesviruses, those in the

BHV-4 group are the most similar to AHV-1; they have the highest level of serological cross-reactivity and similar DNA restriction endonuclease patterns (33). Pseudorabies, also known as Aujesky's disease, infects a variety of species, but domestic swine are thought to be the principal reservoir (14). As cattle may be infected with pseudorabies virus, we must also evaluate its serological relatedness to AHV-1.

Virus neutralization is the only accepted asay for providing a definitive diagnosis of AHV-1 infection (19,49). This a time-consuming test that can only be done at only a few laboratories certified by the United States Department of Agriculture to work with the virus under Biohazard level 3 conditions. The assay is currently performed by mixing serial dilutions of test sera with approximately 100 tissue cultures infectious doses (TCID) of WC11 in each test well containing confluent Vero cell monolayers. The test is then read at 10 days and the endpoint (titer) is defined as the dilution of sera that results in prevention of cytopathic effect in 50% of the test wells. An alternate method for quantifying virus is the use of plaque assays. Although undoubtedly more accurate than a $TCID_{50}$ assay, a plaque assay with AHV-1 is more difficult to read and its use has not been readily adopted (17). An ELISA assay would provide a rapid, simple, and sensitive diagnostic test for AHV-1. This assay would not require the use of live virus and could be routinely used by diagnostic laboratories.

An ELISA has been tested by one group for its ability to detect antibodies to AHV-1. Polyclonal serum from gnotobiotic cattle

infected with BHV-1, BHV-2, Movar or SHV-1 did not cross-react in this assay, but the specificity of the ELISA was only 83.9% when tested with bovid sera that may have possessed antibodies to related viral antigens (60,61). An ELISA that utilizes monoclonal antibodies to compete for serum antibodies from infected animals might have a higher degree of specificity, thereby resulting in a lower percentage of false positives.

## Importance of Herpesvirus Membrane Proteins

In order to begin critical study of AHV-1, its pathogenesis, diagnosis and prophylaxis, examination of the virus membrane proteins is pertinent.

Herpesvirus membrane proteins are found on the surface of infected cells and the envelope of the mature virion, and these proteins are important for several reasons. Specific interactions of membrane proteins and host cell receptors are at least one important aspect of cell tropism and host range. It has been demonstrated in Epstein-Barr virus that the large membrane glycoproteins gp350/220 bind to the cellular receptor CR2 (12). Once attachment occurs, the virus nucleocapsid must penetrate the host cell by a fusion event which is also thought to be a function of the envelope proteins (36). The fusion event may occur at the cell surface, which is the case with paramyxoviruses (62), or may occur only after endocytosis of the virion and subsequent fusion of the endocytic vesicle and the virus envelope. This latter mechanism has been shown to occur in vesicular stomatitis virus, Semliki Forest virus, and influenza virus (10,11,62,64). The fusion event in this case is apparently dependent

upon a decrease in pH within the endosome that results in a conformational change of the proteins responsible for the fusion event (59). Once the nucleocapsid enters the cell it is probably transported to the nuclear membrane where DNA is released into the nucleus (4).

Virus membrane proteins are also involved in virus assembly and egress from the host cell (60). High mannose glycoprotein precursors of HSV-1 membrane proteins accumulate at the inner surface of the nuclear membrane, the site of herpesvirus envelopment. It has been suggested that interaction of the glycoproteins and the nucleocapsid or tegument proteins culminates in their aggregation during the process of envelopment (60). Virus is transported through intracytoplasmic membranes to the cell surface. Further processing of the glycoproteins occurs within the Golgi, and interactions between the glycoproteins and intracytoplasmic membranes are thought to direct egress of the virus from the cell, although the exact mechanism is not known (56). Although these processes have not yet been studied in AHV-1, similar mechanisms of virus/host cell interactions probably exist.

Animals that are naturally infected with herpesviruses tend to have high antibody titers to various membrane proteins, and these antibodies often posses virus-neutralizing activity <u>in vitro</u>. It has also been demonstrated that passive transfer of antibody to the glycoproteins of HSV confers protection to mice subsequently challenged with an otherwise lethal dose of virus (2).

Virus proteins may thus represent potential reagents for the detection of antibodies in infected animals, as well as potential immunogens for prophylaxis. Further, more detailed characterization of these proteins will provide information useful to better understand the pathogenesis of infection.

# CHAPTER 2

# IDENTIFICATION OF VIRUS-INDUCED PROTEINS

## Introduction

Knowledge and understanding of viral proteins provide a basis for study of infection and pathogenesis, as well as for the development of novel diagnostic reagents and protective immunogens. At the outset of this study little was known and nothing reported concerning the identification and characterization of AHV-1 proteins. It was essential to complete a preliminary analysis of the major virus-induced proteins prior to more detailed characterization of individual virus components.

In order to obtain a profile of the major viral induced proteins and an indication as to when after infection these various proteins were produced in the largest amounts, a series of time course studies of AHV-1 infected cells was done. Not only did these experiments allow us to gain an appreciation of relative molecular weights of virus induced proteins that are metabolically labeled with $^{35}S$-methionine, but they also provided two additional pieces of information. We determined the point during the virus-replication cycle individual polypeptides were produced in the highest concentrations, and indentified the times we might maximize radiolabeling of particular proteins of interest. Since glycoproteins present in cell and virus membranes are highly immunogenic and

responsible for initiating a virus neutralizing antibody response, later experiments focused on their identification (1,59).

## Materials and Methods

### Cells and Virus

The WC11 strain of AHV-1 originally isolated in 1960 by Plowright and coworkers (44) was grown in Vero (African green monkey kidney) cells. Cells were infected at multiplicities of infection (MOI) of 1 plaque-forming unit (PFU) to 100-1000 cells. Higher multiplicities of infection were difficult to obtain, as yields of infectious virus were low. Virus was allowed to absorb to the cells for 2 hours, and then was overlaid with Dulbecco's minimal essential medium (DMEM,Gibco) supplemented with 100 IU/ml penicillin and 100 ug/ml streptomycin and 2% heat-inactivated fetal bovine serum. Infection was allowed to proceed at 34°C until cytopathic effect was extensive and the cell monolayer no longer adhered to the plate. Cells were pelleted at 1500 rpm for 10 minutes, and the supernatant removed. The supernatant provided stock virus for further experiments. The cells were stored at -70° C for later use as immunogen, or as antigen in ELISA assays.

Strain 1982 of AHV-1 was obtained from the San Diego Zoo and grown in primary bovine embryonic lung (BEL) cells. Infection and harvest of virus were done as for WC11. Multiplicities of infection of 5-10 were possible as yields of infectious virus were approximately 1000 fold greater with the 1982 strain than with the WC11 isolate. Virus titers were determined by counting the number of plaques formed

in Vero cell monolayers at 10 days post infection. Titers of WC11 ranged from $1.5 \times 10^3$ to $1 \times 10$ PFU per ml. Titers of 1982 pools ranged from $1 \times 10^6$ to $1 \times 10^7$ PFU per ml.

Radiolabeling

Vero cells were infected with WC11 virus at an MOI of 1 PFU to 100-1000 cells, and labeled with $^{35}$S-methionine or $^{3}$H-glucosamine in minimal amounts of media containing 1/10 the normal concentration of methionine or glucose. Replicate cultures of infected cells were labeled at 24-hour intervals from days 1 through 10 postinfection. At the end of each labeling period cells were scraped off with a rubber policeman into ice-cold, phosphate-buffered saline (PBS), pelleted by centrifugation, washed two times in PBS, and the pellets stored at -20°C. Bovine embryonic lung cells were infected with strain 1982 virus at an MOI of 5-10 PFU/cell and labeled with $^{35}$S-methionine between 24 and 48 hours postinfection. Cells were harvested as described above for WC11-infected samples above.

Virus that was to be extrinsically labeled with $^{125}$I was concentrated from spent culture media by centrifugation at 100,000 g. Virus harvested from 50 ml of spent culture media was suspended in PBS and labeled with 0.5-1.0 mCi of $^{125}$I by the use of Iodogen (tetrachlorodiphenylglycouril; Pierce Chemical Co.) (13). Free iodine was removed by sieving on a 5 ml desalting column (P6-DG;Biorad). Efficacy of labeling was determined by comparing the radioactivity present in trichloroacetic acid insoluble material with total counts in 10 $\mu$l samples.

Labeled samples were either suspended immediately in 100 $\mu$l sample buffer containing 0.65 M Tris, 1% sodium dodecyl sulfate (SDS), 10% glycerol and 1% 2-mercaptoethanol, or prepared for immunoprecipitation.

Antibody Production

Polyclonal antibody to AHV-1 was prepared by repeated immunization of a rabbit. For the first injection, approximately $10^8$ infected cells were suspended in 2.0 ml of PBS, emulsified with an equal volume of complete Freund's adjuvant, and injected at multiple subcutaneous sites of a young adult female rabbit. Freund's incomplete adjuvant was used for all subsequent immunizations. The rabbit was injected three times at biweekly intervals with infected cell lysate, and 3 times at biweekly intervals with virus concentrated by centrifugation from 100 ml of spent culture media. After the immunization schedule was completed, the rabbit was sedated with 0.1 ml/kg of Innovar-vet (fentanyl 0.04% and droperidol 2%) and bled from the central auricular artery.

Antibody was purified from serum by affinity chromatography on protein A-agarose. Purified antibody was then repeatedly absorbed at 4°C for 24 hours with approximately 5 x $10^8$ Vero cells and repeatedly passed over two sets of affinity columns, the first composed of Vero cell proteins coupled to cyanogen bromide activated Sepharose, and the second comprised of bovine testicle cell proteins coupled to the same matrix. The rabbit antibody obtained by these procedures was initially highly reactive with bovine serum albumin in an ELISA assay and efficiently immunoprecipitated a very heavy band of an $^{125}I$

labeled protein of approximately 70 kDa. Further absorption over an affinity column made of BSA coupled to Sepharose beads reduced the ability to immunoprecipitate the 70 kDa protein. The antibody was chromatographed on protein-A agarose and the protein concentration determined with BCA Protein Assay (Pierce Chemical Co.)

Immunoprecipitation

Pellets of radiolabeled cells or viruses that were to be immunoprecipitated were solubilized by suspension in radio-immunoprecipitation buffer (RIPA), containing 0.05 M Tris hydrochloride pH 7.2. 0.15 M NaCl, 0.1% deoxycholate, 1.0% Triton X-100, 0.1% SDS, 100 U aprotinin per ml and 0.1 M phenylmethyl-sulfonylflouride (PMSF), and sonicated for 30 seconds. Sonicated material was then centrifuged at 100,000 g for 1 hour to remove the non-soluble portion of the lysate. Four hundred microliter samples of the lysate were added to 125 $\mu$l of protein A-agarose beads (Genzyme Corp., Boston, Massachusetts) and 100 $\mu$g of purified R21 rabbit antibody and rocked on a platform at 4 C for 3 hours. The agarose beads were then pelleted by centrifuging in a Eppendorf centrifuge for 4 min, washed 4 times with RIPA buffer and boiled for 3 min in 105 $\mu$l of sample buffer to dissociate immunoprecipitated proteins. The amount of radioactivity in a 5 $\mu$l sample was determined and the proteins were either immediately analyzed or stored at -20 C for analysis at a later time.

Electrophoresis

Immunoprecipitated samples were analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (30). Gels consisting of 0.65 M Tris pH 8.8, 9% acrylamide, 0.1% SDS, 0.1% glycerol, 0.83% ammonium persulfate, and 0.28% N,N'diallyl-tartardiamide (DATD) were poured, overlaid with 1-butanol and allowed to polymerize for at least 2 hours. After polymerization, the gels were washed with 0.65 M Tris pH 6.8 and stacking gels consisting of 0.65 M Tris pH 6.8, 4% acrylamide, 0.1% SDS, 0.05% glycerol, 0.83% ammonium persulfate, and 0.28 % DATD were poured and allowed to polymerize for 45 minutes. Immunoprecipitated samples and molecular weight standards were loaded into individual wells in the stacking gels and submerged in reservoir buffer of 25 mM Tris and 200 mM glycine with 1% SDS. A constant voltage of 50 V was applied for electrophoretic separation of proteins. Gels were stained with 0.1% Coomassie blue stain for 5 minutes and destained with several changes of destain (7% methanol and 7% acetic acid) over a 12- to 24-hour period to visualize the molecular weight standards. Gels containing proteins labeled with $^{125}I$ were dried on filter paper and placed in contact with XAR film (Eastman Kodak Co., Rochester, N.Y.) at -70 C for fluorography. Gels containing $^{35}S$ or $^{3}H$ labeled proteins were infused with 2,5-diphenyloxazole (PPO) by treating the gel in two 30 min changes of dimethyl sulfoxide (DMSO) followed by 22% PPO in DMSO for 3 hours. The PPO was precipitated in the gel by soaking in $dH_2O$ for at least 1 hour and dried on filter paper. The gels were then placed in contact with XAR film at -70 C for fluorography (5).

# Results

## Time course experiments

Replicate cultures of infected cells were labeled at 24-hour intervals from days 1 through 10 postinfection. By 10 days post infection 100% of the cell monolayer was damaged by virus infection and by day 11 postinfection, the majority of cells were sloughed from the surfaces of the tissue culture flasks. At the end of each labeling period, cells were harvested as described and prepared for electrophoresis. Identification of virus induced proteins was complicated by continuation of host cell protein synthesis (Fig 2.1); host cell proteins continued to be made in large amounts even in the face of 100% cytopathic effect (CPE) at 10 days postinfection. However, upon close examination at least 4 virus-induced proteins could be distinguished. These four proteins had molecular weights of 145,000, 135,000, 125,000, and 110,000. It was also apparent that each of these proteins increased over time, being first readily visible at day 6 and reaching maximum amounts at day 10 postinfection (Figure 2.1).

In order to visualize virus induced proteins more clearly, a portion of the $^{35}S$-methionine labeled AHV-1 infected cell lysate from each 24-hour postinfection time interval was immunoprecipitated with rabbit anti-AHV-1 antibody (Figure 2.2). Although host cell proteins continued to complicate interpretation, it was now possible to discern a minimum of 15 virus induced proteins, ranging in molecular weight from 32,000 to 180,000. In an effort to determine the relationships of molecular weights of proteins specified by strain 1982 and strain

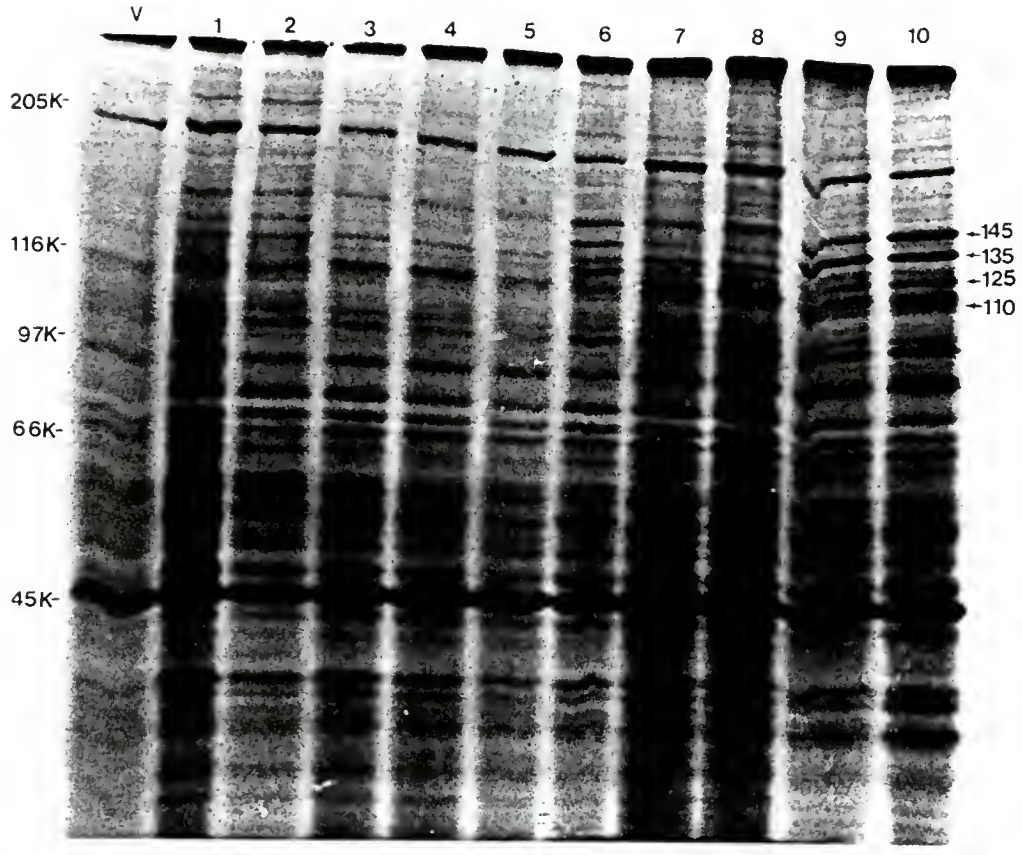


Figure 2.1 - SDS-PAGE analysis of $^{35}$S-methionine labeled proteins in mock infected Vero cells harvested after labeling between 0 and 24 hours post infection (V) and AHV-1 infected Vero cells harvested at the end of each of 10 consecutive 24-hour labeling periods between 0 and 10 days post infection (1-10). Numbers to the right indicate virus induced proteins and their apparent molecular weights (x1000).

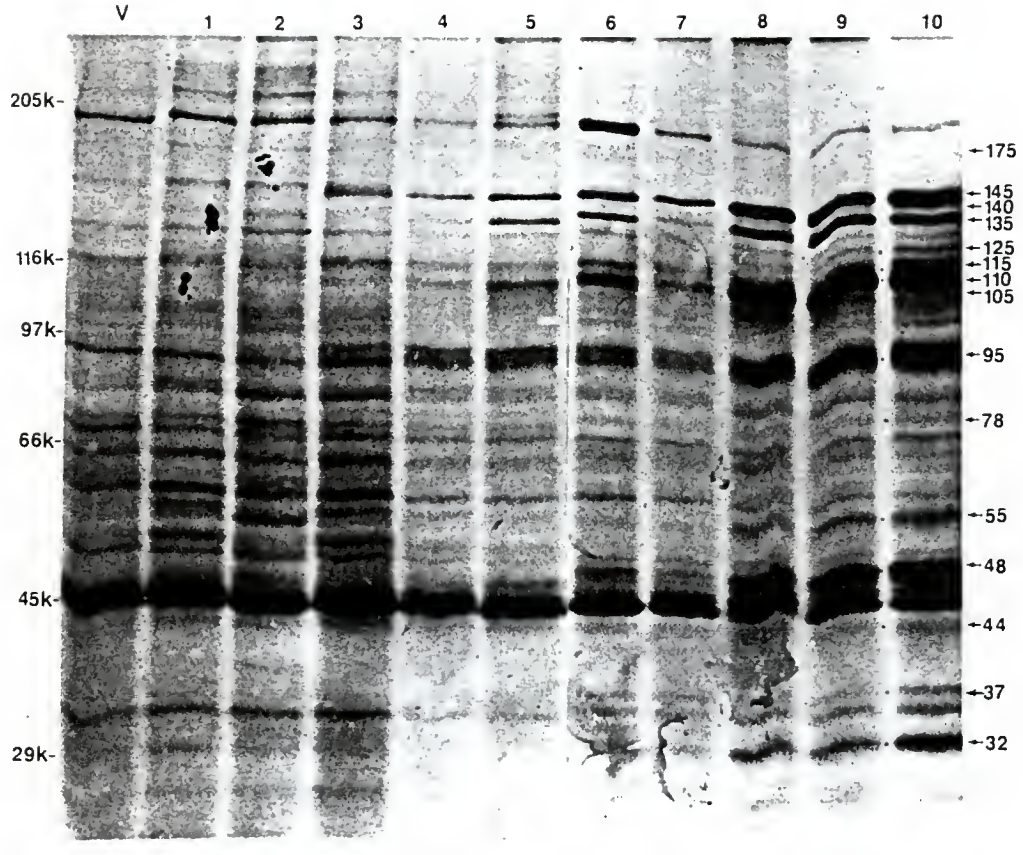


Figure 2.2 - SDS-PAGE analysis of $^{35}$S-methionine labeled proteins immunoprecipitated by rabbit anti-AHV-1 antibodies from mock infected Vero cells harvested after labeling between 0 and 24 hours post infection (V), and AHV-1 infected Vero cells labeled for 24-hour intervals for 10 days after infection (1-10); mock infected and infected cell lysate used in this experiment were also used in the experiment depicted in Figure 2.1. Numbers to the right indicate virus induced proteins and their apparent molecular weights (x1000).

WC11, cells were infected with each strain, labeled with $^{35}S$-methionine, harvested and immunoprecipitated with polyclonal rabbit antibodies as described above. The $^{35}S$-methionine labeled protein profiles of WC11 and 1982 are virtually identical with one exception. A 37 kDa protein is immunoprecipitated from WC11-infected Vero cell lysate, but not from lysate of 1982-infected BEL cells. All other viral induced proteins identified in WC11 appear to have counterparts in the 1982 strain with similar molecular weights (Figure 2.3).

Identification of Membrane Proteins

Our primary interest was in the membrane proteins of AHV-1, and to begin to identify these, WC11 virions were extrinsically labeled with $^{125}I$, lysed, immunoprecipitated with polyclonal immune serum and analyzed by SDS-PAGE. Six virus-specific proteins were identified that were not visible in labeled lysate of mock infected Vero cells. These proteins ranged in molecular weight from 48,000 to 145,000 (Figure 2.4).

Strain 1982 was also extrinsically labeled with $^{125}I$ lysed in RIPA and the lysate immunoprecipitated with rabbit polyclonal antibodies to AHV-1 (R21). A pattern of bands representing proteins of molecular weights similar to those of WC11 was evident (Figure 2.4). These virus proteins ranged in molecular weights from 32,000 to 130,000. One notable difference between the 2 strains in $^{125}I$ labeling experiments was the lack of reactivity of R21 antibodies with 1982 surface protein of 95 kDa, and the reactivity of R21 with a diffuse band of 32 kDa in strain 1982 that was not seen in WC11

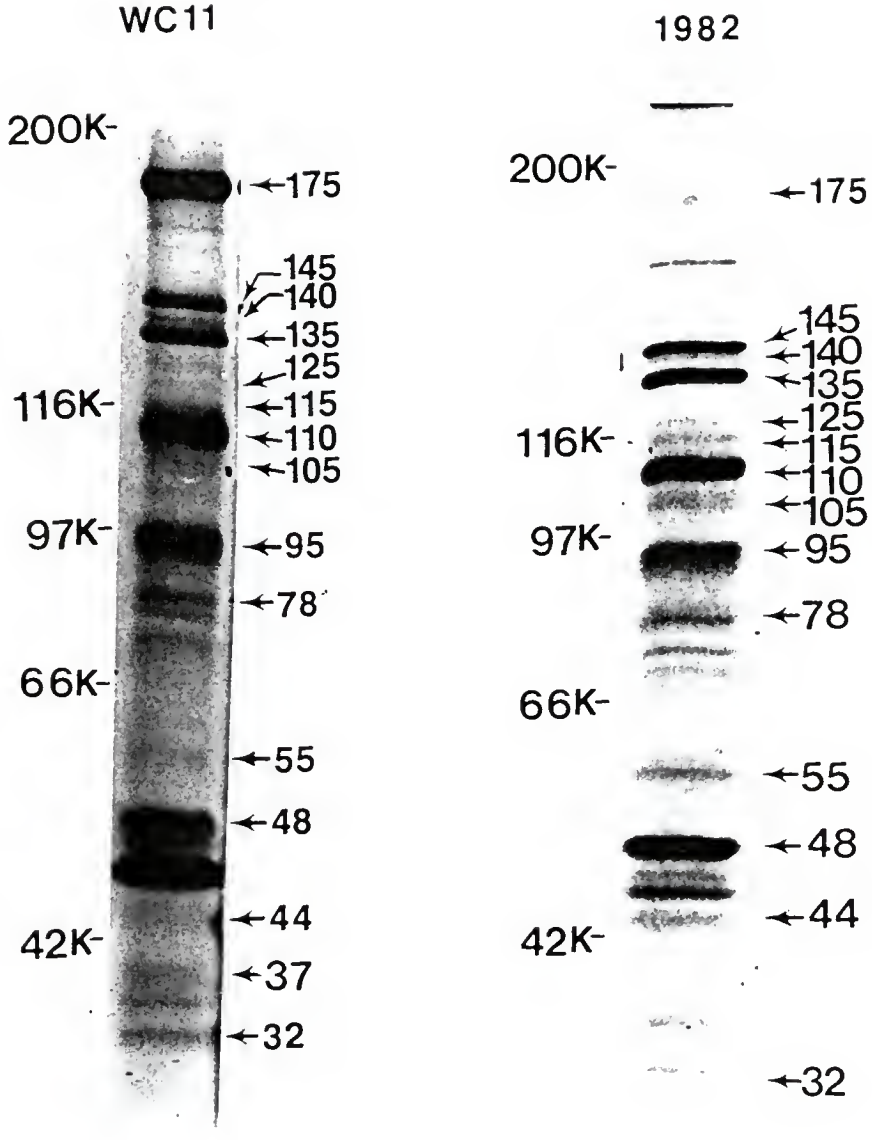


Figure 2.3 - SDS-PAGE analysis of $^{35}$S-methionine labeled proteins immunoprecipitated by rabbit anti-AHV 1 antibody from AHV-1 strain WC11 (WC11) and strain 1982 (1982) infected cells. Numbers to the right indicate virus induced proteins and their apparent molecular weights (x1000).

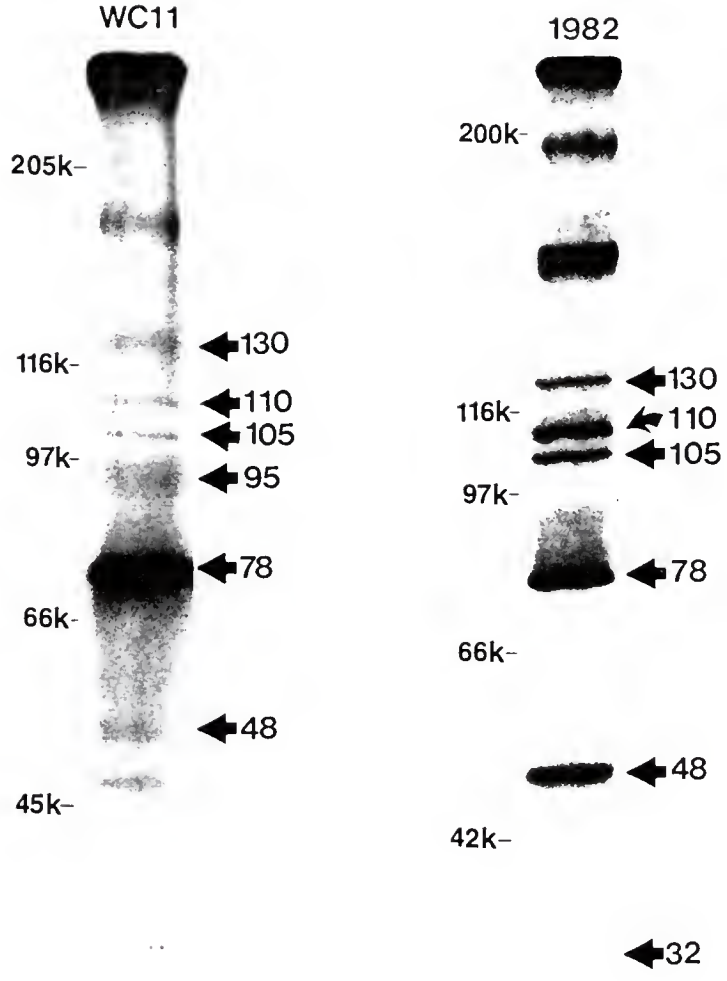


Figure 2.4 - SDS-PAGE analysis of $^{125}I$ labeled proteins immunoprecipitated from extrinsically labeled AHV-1 strain WC11 (WC11) and strain 1982 (1982) labeled virions. Numbers to the right indicate virus proteins and their apparent molecular weights (x1000).

(Figure 2.4). Proteins of 130 kDa, 110 kDa, 105 kDa, and 48 kDa were readily identified when either strains WC11 and 1982 were labeled with $^{125}I$ (Figure 2.4). Also seen in both strains was a protein with a molecular weight of 175,000. This protein is probably cellular in origin, as monoclonal antibodies were produced that immunoprecipitated an abundant protein of similar molecular weight from both uninfected BEL and Vero cells (see below).

Many membrane proteins of herpesviruses are glycosylated. It was therefore of interest to determine which of the molecules that labeled with $^{125}I$ also contained sugars. Vero cells were infected with WC11 and labeled with $^{3}H$-glucosamine for 24 hours at day 10 post infection Immunoprecipitation with rabbit anti-AHV-1 antibody and analysis by SDS-PAGE (Figure 2.5) allowed identification of ten virus induced glycoproteins with molecular weights of 145,000, 130,000, 115,000, 110,000, 105,000, 95,000, 78,000, 72,000 and 48,000 and 32,000. Polyclonal rabbit antibody immunoprecipitated a nearly identical pattern of proteins from 1982 infected cells labeled with $^{3}H$-glucosamine. One minor difference was the presence of a 62 kDa glycoprotein in cells infected with 1982 and the lack of a 72 kDa protein identified in Vero cells infected with WC11. A comparison of $^{35}S$-methionine labeled proteins from WC11 and 1982 infected cells was also made. An almost identical pattern of proteins was seen with each strain (Figure 2.3).

A summary of WC11 and 1982 induced proteins thus far identified by labeling with various radioisotopes is given in Table 2.1.

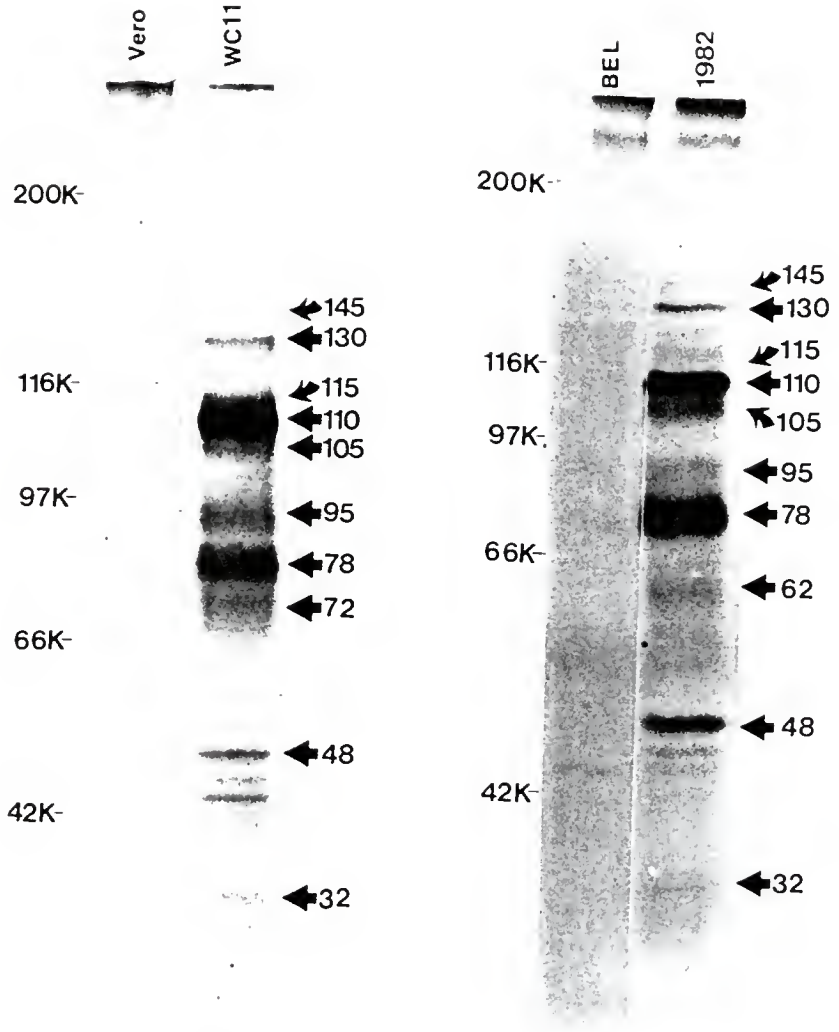


Figure 2.5 - SDS-PAGE analysis of $^{3}$H-glucosamine labeled proteins immunoprecipitated from uninfected (Vero, BEL) and AHV-1 strain WC11 (WC11) and AHV-1 strain 1982 (1982) cells. Numbers to the right indicate virus induced proteins and their apparent molecular weights (x1000).

Table 2.1- Proteins Identified by Immunoprecipitation with Polyclonal Antibodies to AHV-1.

| M.W.[a] | RADIOLABEL | | | | | |
|---|---|---|---|---|---|---|
| | $^{35}$S-methionine | | $^{125}$I | | $^{3}$H-glucosamine | |
| | WC11 | 1982 | WC11 | 1982 | WC11 | 1982 |
| 175 | + | + | | | | |
| 145 | + | + | | | + | + |
| 140 | + | + | | | | |
| 135 | + | + | | | | |
| 130 | | | + | + | + | + |
| 125 | + | + | | | | |
| 115 | + | + | | | + | + |
| 110 | + | + | + | + | + | + |
| 105 | + | + | + | + | + | + |
| 95 | + | + | + | | + | + |
| 78 | + | + | + | + | + | + |
| 72 | | | | | + | |
| 62 | | | | | | + |
| 55 | + | + | | | | |
| 48 | + | + | + | + | + | + |
| 44 | + | + | | | | b |
| 37 | + | | | | | |
| 32 | + | + | | + | + | + |

a- Molecular weight (x1000).
b- A glycoprotein of 44 Kd was not precipitated with polyclonal antibody, but was identified with monoclonal antibody 11C3 (see Chapter 4).

## Discussion

It was readily apparent that host protein synthesis continues relatively unabated throughout infection with AHV-1. A total of only 14 virus induced proteins could be identified by metabolically labeling cells with $^{35}$S-methionine. The genome of AHV-1, however, is approximately 8 x $10^6$ daltons, and it is probable that its total coding capacity is far greater than this experiment would suggest (31). We assume, therefore, that some virus transcripts are either translated in amounts too small to be detected by protocols used, that they have mobilities greater or smaller than the ranges analyzed on gels, or that the translation products are poorly labeled with $^{35}$S methionine. The experiment did, however, allow us to conclude that metabolic labeling of WC11 induced proteins for further analysis was optimally done at 10 days post infection.

Our long-term objective was to characterize the envelope proteins of AHV-1. Six virus proteins were immunoprecipitated with rabbit polyclonal antibodies from virions extrinsically labeled with $^{125}$I. All six of these proteins were also glycosylated and therefore were presumed to be surface glycoproteins. Two interesting differences were found between WC11 and 1982 extrinsically labeled with $^{125}$I. A surface-labeled protein of 95 kDa was identified in WC11 but lacking in the strain 1982, whereas a protein of 32 kDa was exclusive to strain 1982. In contrast, proteins of identical size were visible, although not prominent, in analyses of material from cells infected with both strains of virus if they had been labeled with $^{3}$H-glucosamine or $^{35}$S-methionine. If we assume that the proteins

labeled by these techniques are identical, then we suggest that they may in fact be molecules that are not actually found on the virus surface. Their apparent presence on the surface of the virion may then be a result of damage to the virions, allowing access to normally internal proteins and subsequent labeling with $^{125}I$. It is also conceivable that the proteins are actually found on the surface of one strain but not the other, or that the portions of each molecule possessing tyrosines reactive in the labeling process are differentially accessible.

More extensive characterization of the envelope proteins required monoclonal rather than polyclonal reagents. The next step was to prepare such reagents.

# CHAPTER 3

# MONOCLONAL ANTIBODIES

## Introduction

Monoclonal antibody technology, as originally described by Kohler and Milstein (29), has provided invaluable tools for the study of proteins. Monoclonal reagents represent a distinct advantage over polyclonal reagents for a variety of reasons. Of particular relevance to our work was the fact that since monoclonal antibodies are specific for a single epitope, or at least a very limited number of cross reactive epitopes, they can be used to isolate and analyze individual proteins within a mixture. Our goal was to prepare monoclonal reagents that would allow us to identify and characterize individual envelope proteins of AHV-1. In addition, we were able to use the antibodies we produced to identify at least one protein that can induce a virus-neutralizing response. These antibodies also provided the basis of a diagnostic assay for AHV-1 infection that is described in Chapter 5.

## Materials and Methods

### Mouse Immunizations

Virus was concentrated by ultracentrifugation from 50 ml of spent culture media and used for subcutaneous immunization of 3 Balb/c mice. The virus was emulsified in an equal volume of complete Freund's adjuvant to give a final volume of 0.5 ml per mouse. Mice were

subsequently immunized at 1-2 week intervals by subcutaneous injection with a similar amount of virus emulsified in incomplete Freund's adjuvant. After three injections a small amount of blood was taken from the tail veins of the mice and sera separated from the blood were tested in surface immunofluorescence assays for the presence of antibody to AHV-1 induced surface proteins. When mice tested positive by this assay, they were boosted by intraperitoneal injection with 0.5 ml of antigen in PBS and sacrificed 3 days later for preparation of hybridomas.

Fusion

Hybridomas were made by fusing mouse spleen cells with the myeloma cell line SP2/0 Ag 14. Mice were anesthetized with methoxyflurane and exsanguinated by cardiac puncture using a 23-gauge needle. The spleen was then removed and aseptically placed in sterile DMEM supplemented with non-essential amino acids, sodium pyruvate, 0.1 M Hepes and 20% fetal bovine serum (SDMEM). The spleen cells were released from the spleen capsule by grinding the spleen between two frosted-end microscope slides. Red blood cells were lysed with ammonium chloride and spleen cells washed twice with SDMEM. Hyperimmune spleens yield more than $10^8$ white blood cells. The spleen cells were combined with the SP2/0 myeloma cells at a ratio of five spleen cells to one SP2/0 cell, washed in serum-free SDMEM and fused together with 50% polyethylene glycol 1500 for one minute. Serum-free media was slowly added and the fused cells were washed to remove PEG and plated into 96 tissue culture plates at $1 \times 10^5$ cells in 100 $\mu$l

SDMEM with 20% fetal bovine serum (FBS) per well. After 24 hours, 100 $\mu$l of SDMEM containing two times the normal concentration of hypoxanthine, thymidine and aminopterin was added to each well. Cells were fed when media was depleted. Outgrowths were screened for the production of antibody and transferred first into 24 well tissue culture plates, and then into petri dishes for further growth. When cultures had expanded to approximately 5 million cells they were frozen in SDMEM containing 20% FBS and 10% dimethyl sulfoxide.

ELISA

Culture media from hybridomas were initially screened by an enzyme linked immunosorbent assay that used WC11-infected Vero cells as antigen. Virus-infected cells were collected when virus CPE was approaching 100%. Infected cells and uninfected control cells were pelleted by centrifugation, suspended in carbonate/bicarbonate buffer pH 9.6 and lysed by freeze thawing three times. These antigen preparations were then tested at various dilutions to determine the concentrations that produced the maximum differential signal between infected and non-infected cells when polyclonal mouse anti-AHV-1 antibody was reacted with each. Ninety-six well flat bottomed plates were incubated overnight with varying amounts of antigen in carbonate/bicarbonate buffer 9.6. Plates were then incubated sequentially with 5% skimmed milk in PBS-Tween 20, dilutions of mouse antibody, goat anti-rabbit or anti-mouse antibody conjugated to horse radish peroxidase (Organon Teknika Corp., Durham, N.C.) and the substrate orthophenylenediamine (Sigma). Plates were washed between

each incubation with 0.05% Tween 20 in 0.85% NaCl; the final enzyme reaction was stopped with 2 N $H_2SO_4$ and analyzed for color change at 492 nm.

Surface Fluorescence

Vero cells that had been infected with WC11 10 days previously, were placed at 4°C for 30 minutes and removed from tissue culture flasks by vigorous pipetting. Cells were pelleted by centrifugation, fixed with 0.1% paraformaldehyde, washed three times with PBS, and incubated with culture media that had been concentrated to one-tenth their original volume by dialysis against polyethylene glycol. Cells were then washed three times in PBS, incubated for 30 minutes with fluorescein conjugated rabbit anti-mouse antibody, washed with PBS and examined with a fluorescence microscope.

Virus Neutralization

Hybridomas were screened for their ability to neutralize virus in a standard plaque reduction assay. A known number of PFU were incubated for one hour at 37°C with hybridoma culture media that had been concentrated 10 fold by dialysis against polyethylene glycol. The mixture was titrated in confluent Vero cell monolayers in 24 well plates. Virus was allowed to absorb for 2 hours, at which time each well was overlaid with 1 ml of SDMEM containing 2% fetal bovine serum. Infection was allowed to proceed for 10 days, at which time the number of plaques in the cell monolayer was counted. Hybridoma culture media that reduced the number of plaques by 50% were determined to possess

neutralizing activity. Subclones of hybridoma 12B5 were further screened with this technique to confirm neutralizing activity.

## Results

### ELISA

The difference in reactivity of mouse anti-AHV-1 sera with infected and uninfected Vero cells was greatest if ELISA plates were coated with 30 $\mu$g of cell protein. The difference in reactivity between infected and uninfected cell proteins did not begin to diminish appreciably until a protein concentration of less than 3 $\mu$g per well was used (Figure 3.1) Three micrograms of protein per well were used in all subsequent ELISA assays. Non-immune mouse serum reacted at equally low levels with infected and non-infected Vero cells. This indicated the specificity of this ELISA as a test for anti-AHV-1 antibodies, especially at the higher concentrations of antigen.

### Hybridoma screening

After several unsuccessful fusions, one fusion resulted in outgrowth in a total of 165 wells of five 96 well culture dishes. Culture supernatant was harvested from the 96 well plates, and tested in the ELISA assay. Of the 165 hybridomas tested, a total of 97 tested positive by ELISA. A positive reaction was determined to be one in which the optical density in wells coated with infected cell antigen was at least 0.300 and approximately 2 times higher than the optical density in wells coated with uninfected cell lysate. The 97 hybridomas positive by at least two of these criteria were then screened for their ability to bind surface proteins of infected but

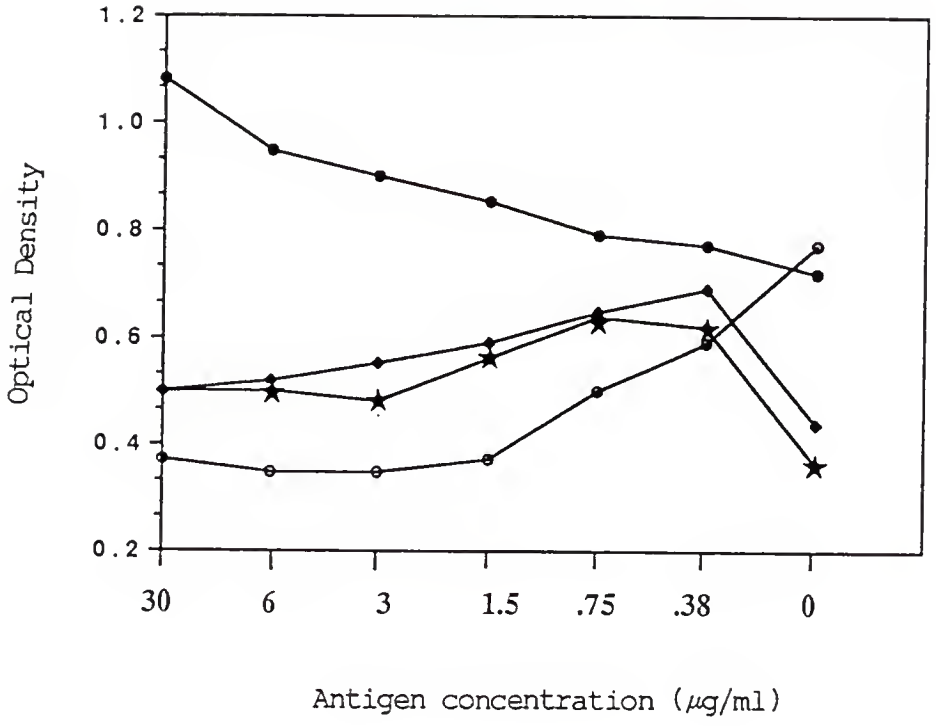


Figure 3.1 - Titration curve of AHV-1 infected and uninfected Vero cell ELISA antigen. ● - Polyclonal mouse anti AHV-1 serum reacted with AHV-1 infected cell lysate. O -Polyclonal mouse anti-AHV-1 serum reacted with uninfected cell lysate. ◆ - Non-immune mouse serum reacted with AHV-1 infected cell lysate. ★ -Non-immune serum reacted with uninfected cell lysate.

not uninfected cells in an immunofluorescence assay. A total of 56 hybridomas tested positive by these criteria.

Immunoprecipitations with Monoclonal Antibodies

Monoclonal antibodies that were positive by surface fluorescence were screened for their ability to immunoprecipitate virus induced proteins that had been radiolabeled with $^{125}I$ or $^{35}S$-methionine. Hybridomas immunoprecipitated 8 different virus-induced proteins (Table 3.1). Despite previous screening, hybridomas appeared to recognize cell proteins. These antibodies immunoprecipitated molecules of 220 kDa and 180 kDa from both infected and uninfected cells. Hybridomas capable of immunoprecipitating putative surface glycoproteins from both WC11-and 1982-infected cells are described in more detail.

The 145 kDa protein

Five hybridomas were identified that immunoprecipitated a 145 kDa protein. Two of these hybridomas, 14B6 and 41B1 were cloned and culture media containing antibody was concentrated by dialysis against polyethylene glycol. Results of immunoprecipitation with both monoclonal antibodies were identical; therefore only results for 14B6 are shown. The 145 kDa protein was immunoprecipitated with monoclonal 14B1 from virus infected cells labeled with $^{35}S$-methionine (Figure 3.2 and 3.3) or $^{3}H$-glucosamine (Figure 3.6). Antibodies to this protein did not immunoprecipitate a labeled protein from the lysate of $^{125}I$ labeled 1982 or WC11 virions (Figure 3.4 and 3.5). The protein was also not immunoprecipitated by rabbit polyclonal antibodies from $^{125}I$ labeled 1982 virions. It is therefore probable that this protein is not present on the surface of the 1982 virion.

The 95 kDa protein

Eighteen hybridomas were identified that reacted very strongly with a 95 kDa protein induced by strain WC11. Five of these were cloned and concentrated from culture media. Neither the polyclonal rabbit antibody R21 nor monoclonal antibodies 21B1 and 24C1 could immunoprecipitate this 95 kDa molecule from $^{125}I$ labeled strain 1982 (Figure 3.4 and 3.5). All antibodies had similar patterns of reactivity and the results of immunoprecipitating virus are shown only for 21B1. As noted in Chapter 2, a protein of 95 kDa was identified in $^{35}S$-methionine and $^{3}H$-glucosamine labeled virus-infected cells that had been immunoprecipitated with polyclonal rabbit antibodies to AHV-1. Monoclonal antibodies 24C1 and 21B1 did not immunoprecipitate this protein from $^{3}H$-glucosamine labeled 1982-infected cells (Figure 3.6), but did immunoprecipitate a 95 kDa protein from $^{3}H$-glucosamine labeled WC11- infected Vero cells (data not shown). Monoclonal antibody 21B1 also reacted weakly with a protein of 95 kDa from WC11- or 1982-infected cells metabolically labeled with $^{35}S$-methionine (Figure 3.2 and 3.3)

The 44 kDa protein

Three hybridomas were identified as reactive with a protein of an apparent molecular weight of 44,000. One of these hybridomas, 11C3 was cloned and antibody was concentrated from culture media. The 44 kDa protein was labeled with $^{35}S$-methionine and $^{3}H$-glucosamine. It was immunoprecipitated weakly by polyclonal serum, but was readily identifiable after immunoprecipitation with monoclonal 11C3. It was

particularly prominent in infected cells that had been labeled with $^{35}$S-methionine, but could not be identified by labeling of virions with $^{125}$I. The protein was present in both strains of AHV-1 with identical apparent molecular weights.

The 115 kDa protein

Seven hybridomas were found to immunoprecipitate a group of 5 proteins from lysates of cells infected with WC11 or 1982 which had been labeled with $^{3}$H-glucosamine and $^{35}$S-methionine and electrophoresed under reducing conditions. The proteins had molecular weights of 115,000, 110,000, 105,000, 78,000, and 48,000. Two hybridomas reactive with the group, 12B5 and 31B3 were monocloned and used to make ascites in mice. Antibody was purified from ascites by affinity chromatography on protein A-agarose. The patterns of proteins immunoprecipitated by either of these two antibodies were identical, so only analyses of proteins immunoprecipitated with 12B5 are given (Figure 3.2 and 3.3). Monoclonal antibody 12B5 immunoprecipitated only 2 proteins of the group from lysate of $^{125}$I labeled virus, namely those of 78 kDa and 48 kDa. The monoclonal antibody 12B5 had virus neutralizing activity when assessed by a standard plaque reduction assay (Table 3.2). This interesting complex of proteins was therefore selected for closer examination (see Chapter 6).

## Discussion

Production of monoclonal antibodies allows analysis of individual AHV-1 proteins. A summary of the data presented in this chapter

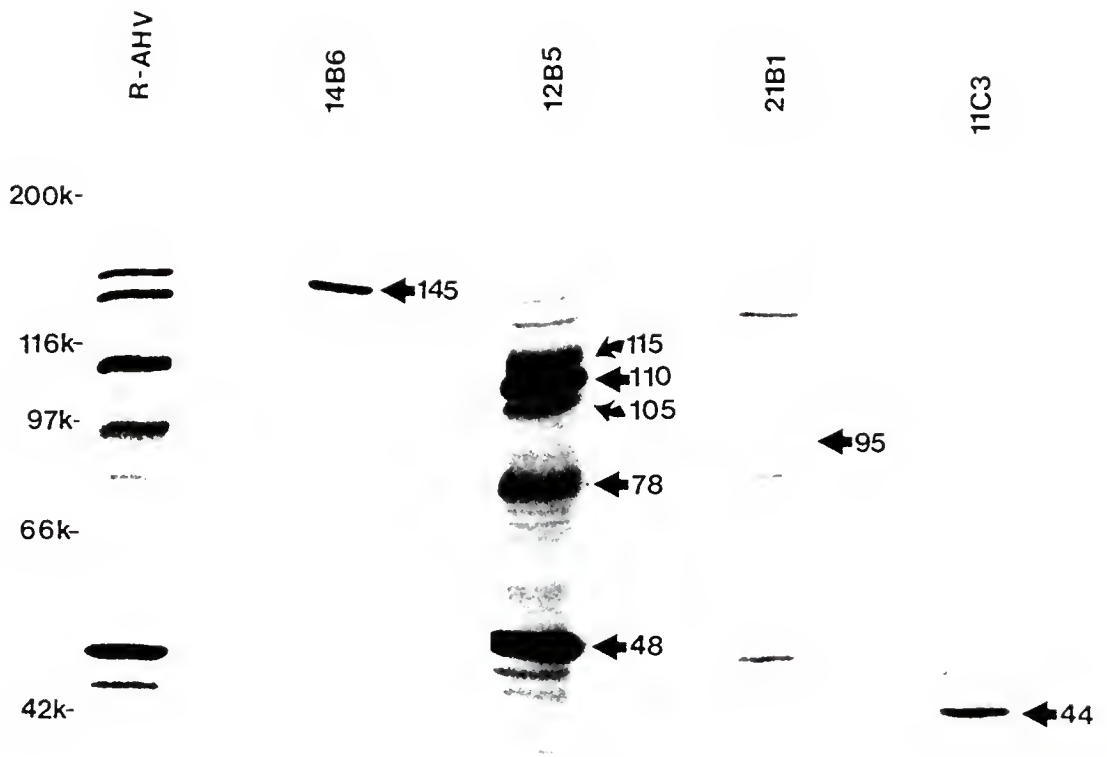


Figure 3.2 - SDS-PAGE Analysis of $^{35}$S-methionine labeled proteins immunoprecipitated from cells infected with WC11 by rabbit polyclonal anti-AHV 1 antibodies (R-AHV), and monoclonal antibodies 14B6, 12B5, 21B1 and 11C3. Arrows to the right indicate proteins immuno-precipitated from infected cells and their apparent molecular weights (x1000).

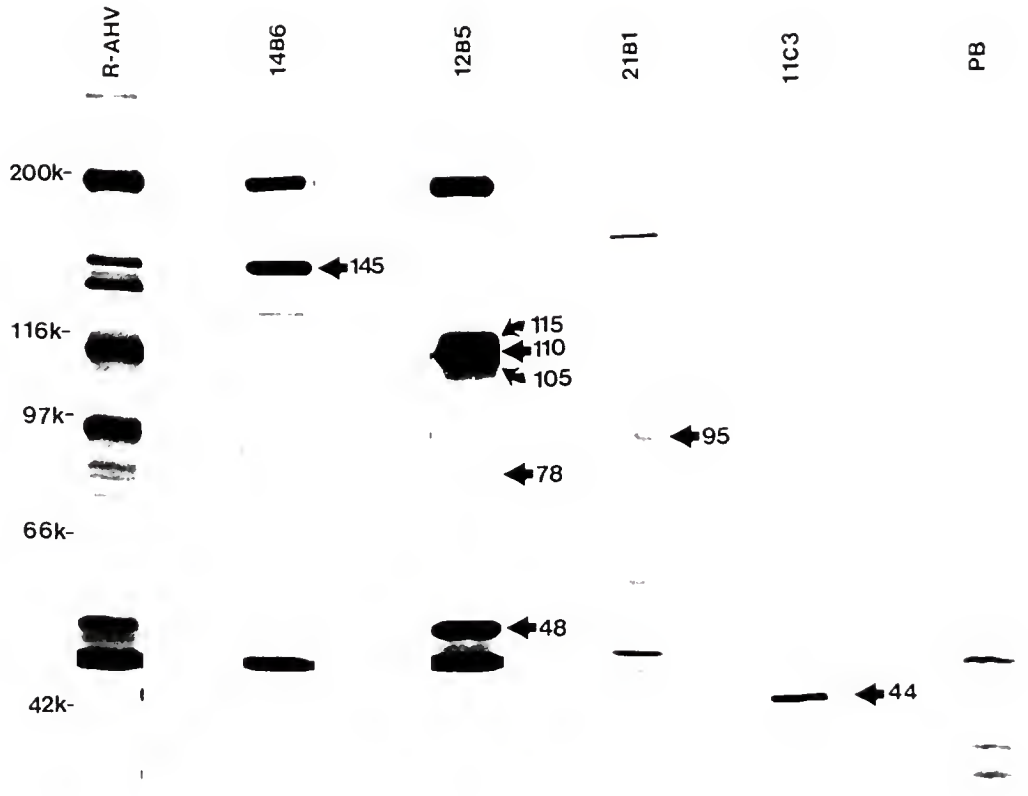


Figure 3.3 - SDS-PAGE analysis of $^{35}$S-methionine labeled 1982 proteins immunoprecipitated by rabbit polyclonal anti-AHV 1 antibodies (R-AHV), non-immune rabbit sera (PB) and monoclonal antibodies 14B6, 12B5, 21B1 and 11C3. Arrows to the right indicate proteins immunoprecipitated from infected cells and their apparent molecular weights (x1000).

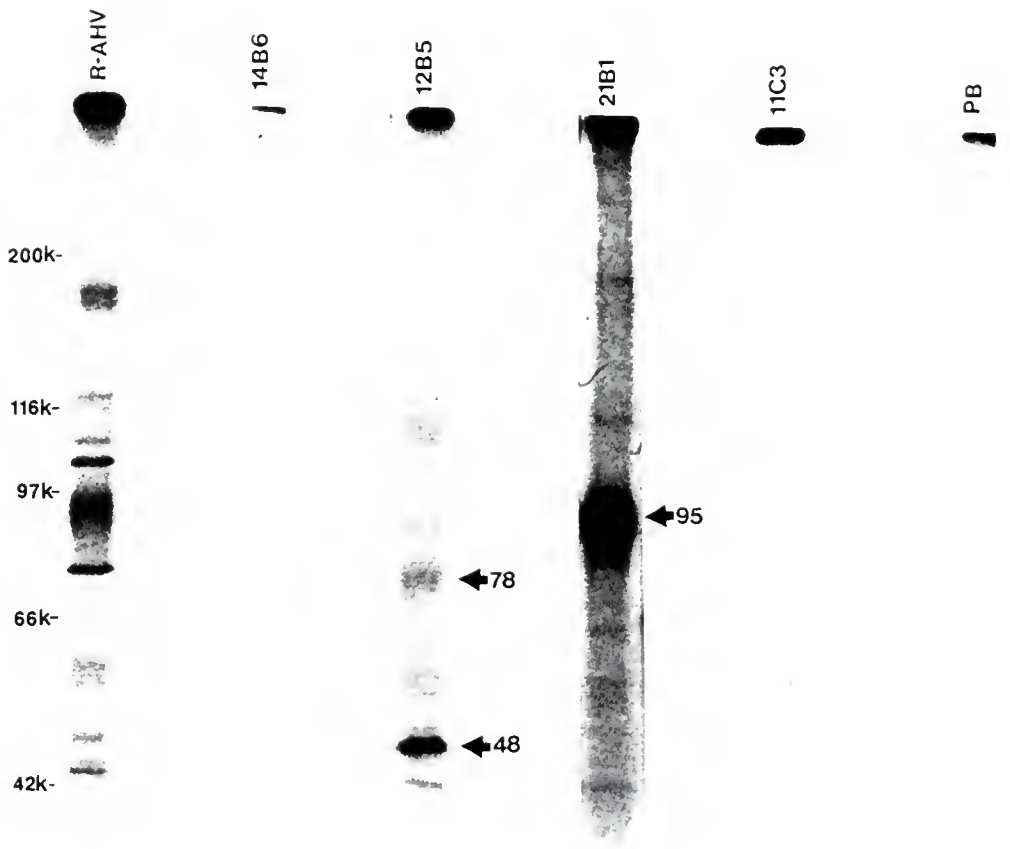


Figure 3.4 - SDS-PAGE analysis of $^{125}I$ labeled proteins of WC11 virions, immunoprecipitated by polyclonal rabbit anti-AHV 1 antibodies (R-AHV), non-immune rabbit sera (PB), and monoclonal antibodies 14B6, 12B5, 21B1 and 11C3. Arrows to the right indicate proteins immunoprecipitated from virion lysate and their apparent molecular weights (x1000).

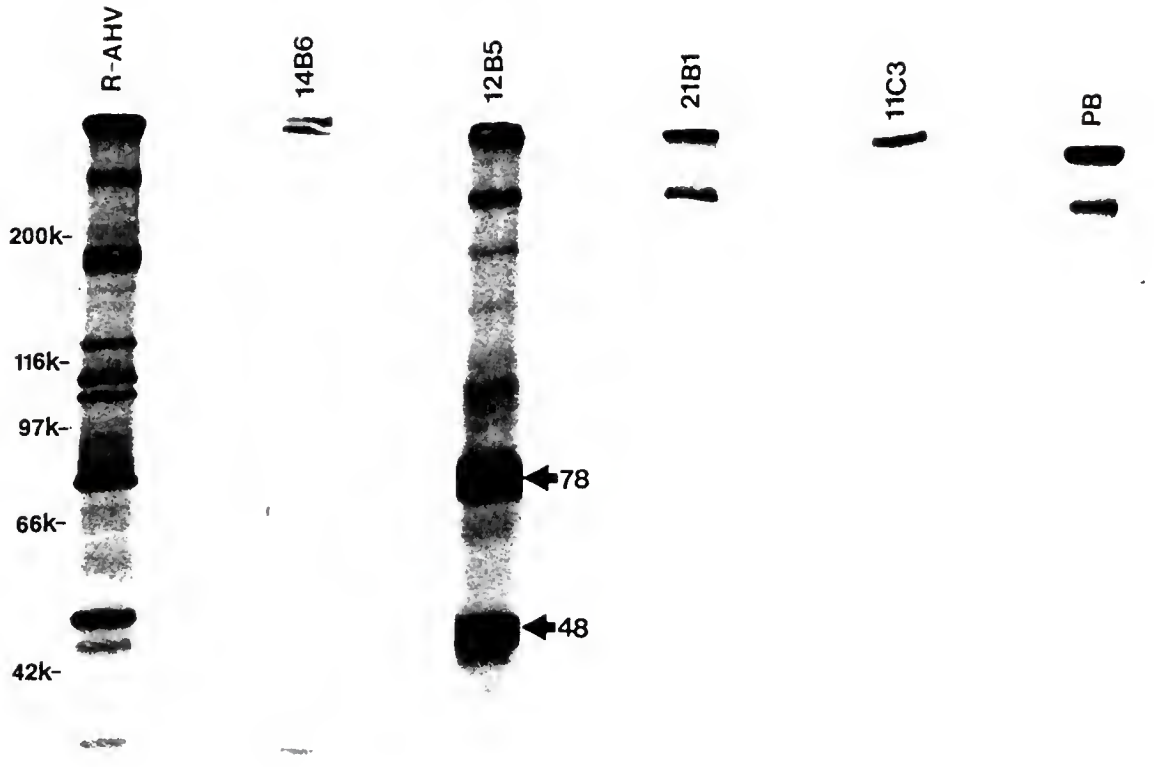


Figure 3.5 - SDS-PAGE analysis of $^{125}I$ extrinsically labeled proteins of 1982 virions, immunoprecipitated by polyclonal rabbit anti-AHV 1 antibodies (R-AHV), non-immune rabbit sera (PB), and monoclonal antibodies 14B5, 12B5, 21B1 and 11C3, Arrows to the right indicate proteins immunoprecipitated from virion lysate and their apparent molecular weights (x1000).

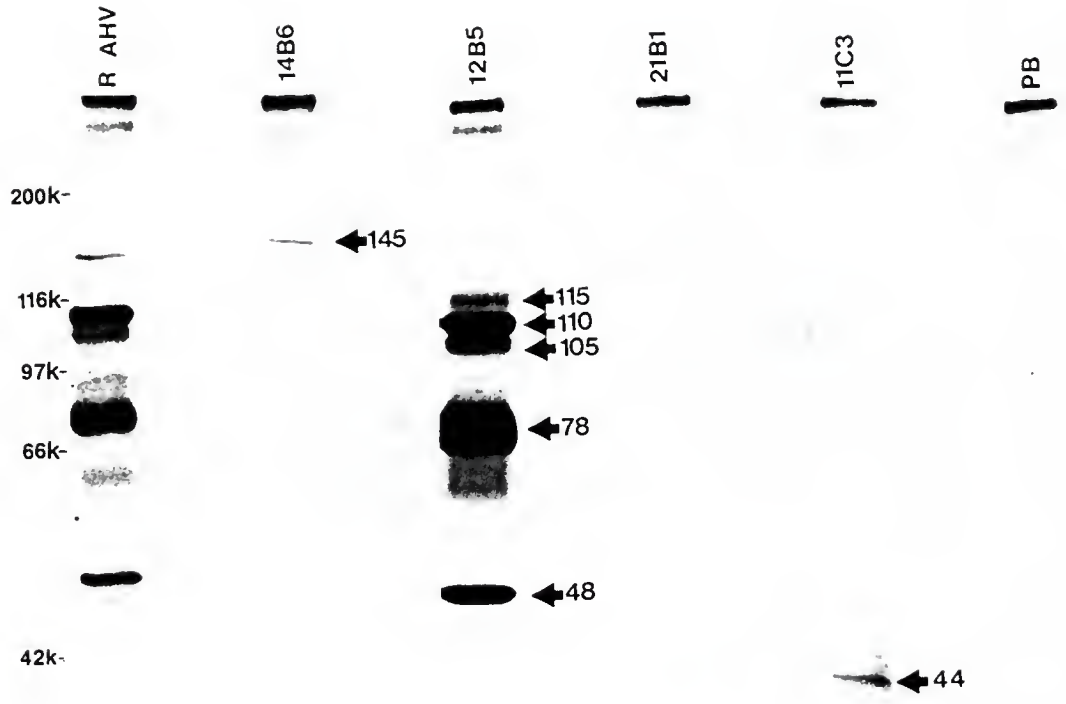


Figure 3.6 - SDS-PAGE Analysis of $^{3}H$-glucosamine labeled 1982 proteins immunoprecipitated by rabbit polyclonal anti-AHV 1 antibodies (R-AHV), non-immune rabbit sera (PB) and monoclonal antibodies 14B6, 12B5, 21B1 and 11C3. Arrows to the right indicate proteins immunoprecipitated from infected cells and their apparent molecular weights (x1000).

Table 3.1 - Summary of Characteristics of Hybridoma Antibodies Reactive with Virus Induced Proteins.

| MW[a] | Hybridoma Antibodies | ELISA Reactivity | | Surface Floresc. | Immunoppt.[b] | Neut.[c] |
|---|---|---|---|---|---|---|
| | | Infected Antigen | Uninfected Antigen | | | |
| 220 | 12C2 | .761 | .208 | + | + | – |
| | 21B2 | .986 | .556 | + | + | + |
| | 22D6 | .490 | .239 | + | + | – |
| | 31D1 m | .456 | .160 | + | + | – |
| 180 | 23C3 m | | | | | |
| | 32D2 | .863 | .247 | + | + | – |
| | 42C5 | .621 | .253 | ND | + | – |
| | 44A5 | .522 | .218 | + | + | – |
| 145 | 11A1 | .390 | .192 | + | + | +/– |
| | 11D3 | 1.559 | .890 | + | + | |
| | 14B6 m | .675 | .227 | + | + | – |
| | 21A2 m | .562 | .151 | + | + | – |
| | 41B1 m | .667 | .354 | + | + | – |
| 115 | 12B5 m | .711 | .116 | + | + | + |
| | 13A6 | 1.486 | .264 | + | + | – |
| | 23A1 | 1.185 | .172 | +/– | + | – |
| | 31B5 m | .828 | .199 | + | + | – |
| | 32A4 | 1.002 | .252 | + | + | – |
| | 22A2 | .599 | .389 | + | + | – |
| | 32D2 | .863 | .247 | + | + | – |

Table 3.1-- continued.

| MW[a] | Hybridoma Antibodies | ELISA reavtivity | | Surface Flouresc. | Immunoppt.[b] | Virus Neut.[c] |
|---|---|---|---|---|---|---|
| | | Infected Antigen | Uninfected Antigen | | | |
| 95 | 11A6 m | 1.452 | .521 | + | + | - |
| | 14D4 | 1.505 | .680 | + | + | - |
| | 21B1 | .448 | .241 | + | + | ND |
| | 21C5 | 1.125 | .267 | +/- | + | - |
| | 21D3 m | .665 | .288 | + | + | ND |
| | 22A3 | .481 | .228 | + | + | ND |
| | 22C6 | .809 | .413 | + | + | ND |
| | 24C1 | .391 | .243 | + | + | - |
| | 24C3 | .429 | .318 | + | + | - |
| | 31A5 | .329 | .174 | + | + | - |
| | 31C3 | 1.931 | 1.062 | + | + | - |
| | 32B3 | .411 | .232 | + | + | ND |
| | 41B1 | .667 | .354 | + | + | - |
| | 41D2 | .456 | .129 | + | + | - |
| | 44D2 | 1.954 | .614 | + | + | - |
| | 22A6 | .379 | .190 | + | + | - |
| | 22B5 m | .555 | .283 | + | + | ND |
| | 31C5 m | .553 | .233 | + | + | - |
| 44 | 11C3 m | 1.532 | .514 | + | + | - |
| | 21B2 | .986 | .556 | + | + | + |
| | 44D2 | 1.954 | .614 | + | + | - |

a - molecular weight of proteins immunoprecipitated by antibodies.
b - positive indicates hybridomas that were capable of immunprecipitating proteins radiolabeled with $^{125}I$ and/or $^{35}S$-methionine.
c - virus neutralizing ability as determined by plaque reduction assay.
m - Indicates hybridomas that have been monocloned.
ND - Indicates not done.

Table 3.2 - Comparison of the Neutralizing Ability of Hybridoma Supernatants, and Known Positive and Negative Sera.

| Experiment 1 | | Experiment 2 | |
|---|---|---|---|
| Antibody | #Plaques | Antibody | #Plaques |
| None | 25 | None | 36 |
| 12C2 | 22 | Goat (-) | 32 |
| 13A6 | 24 | Calf (-) | 34 |
| 21B2 | 11 | 31B1 | 34 |
| 24C3 | 26 | 24B6 | 31 |
| 14C4 | 26 | 21B2 | 23 |
| 44A5 | 21 | 33B2 | 31 |
| 32B2 | 26 | 14E4 | 26 |
| 33A4 | 22 | 33A2 | 34 |
| 44B1 | 20 | 21B6 | 35 |
| 31D1 | 22 | 31C4 | 30 |
| 24C1 | 21 | R21 (+) | 11 |
| 11A1 | 17 | 21A5 | 30 |
| 14B6 | 23 | 11C4 | 24 |
| 11C3 | 22 | 31A4 | 36 |
| 22D6 | 24 | 11A1 | 32 |
| 31C5 | 21 | 12B5 | 9 |
| 21C5 | 21 | 24A4 | 26 |
| 41D2 | 20 | 12A4 | 25 |
| 21A5 | 20 | 24A2 | 33 |
| 23C3 | 25 | 23B3 | 41 |
| 44D2 | 20 | 42A4 | 36 |
| 12B5 | 11 | 41B3 | 32 |
| 31C3 | 28 | 33C4 | 35 |
| 11A6 | 23 | 42D5 | 38 |
| 42C5 | 20 | | |
| 23A1 | 21 | | |
| 22A6 | 25 | | |
| 32D2 | 22 | | |

(-) - Known negative sera
(+) - Known positive sera

appears in Table 3.1. Ninety-five hybridomas were produced to AHV-1 virus induced proteins. Thirteen of these were cloned and the eight proteins of 145, 95, 44, 115, 110, 105, 78, and 48 kDa were more extensively described.

The 145 kDa protein was a glycosylated molecule, but was very poorly labeled with $^{125}I$. Since antibodies to the protein reacted with the surface of infected cells, it is presumably a membrane protein that either merely labeled poorly with the $^{125}I$ or failed to be incorporated into the virion envelope.

The 95 kDa protein was present in virus infected cells although it labeled poorly with $^{35}S$-methionine or $^{3}H$-glucosamine and/or was weakly immunoprecipitated with available hybridoma antibodies. Monoclonal and polyclonal antibodies immunoprecipitated the iodinated 95 kDa protein from strain WC11, but not from strain 1982. This difference could reflect damage to the virion envelope of WC11 and exposure to iodine of proteins that are not normally seen on the surface of the virion or could mean that the protein is differently distributed in the two strains.

The 44 kDa protein was immunoprecipitated from both $^{35}S$-methionine, and $^{3}H$-glucosamine labeled infected cells. This protein was not appreciably labeled by $^{125}I$.

The remaining 5 proteins of 115, 110, 105, 78 and 48 kDa were all precipitated as a group by several monoclonal antibodies. One of the monoclonal antibodies was also capable of neutralizing virus implicating the complex in initiation of infection. It was also noted that not only were the number of plaques reduced by the antibody, but

the plaques appeared to be smaller in diameter. The 5 proteins labeled very well with $^{35}S$-methionine and $^{3}H$-glucosamine in cells infected with either strain WC11 or 1982. They were immuno-precipitated with polyclonal antibodies to AHV-1 and monoclonals 12B5 and 31B3. Monoclonals 12B5 and 31B3 also precipitated at least 2 of the 5 proteins from $^{125}I$ labeled virions. The evaluation of the relationships between the proteins of the group is the subject of Chapter 6.

CHAPTER 4

# RELATIONSHIPS OF AHV-1 WITH OTHER BOVID HERPESVIRUSES

## Introduction

Herpesviruses of man and animals vary widely in their DNA structures, host ranges and growth characteristics. These differences provide the basis for classification into the subfamilies $\alpha$, $\beta$, and $\tau$ (51). The herpesviruses do however share common morphology, mode of entry into host cells (fusion with cell membranes), nuclear location of viral DNA replication and virus assembly, and acquisition of an envelope from the inner nuclear membrane (51). In all herpesviruses examined, virus DNA transcription and protein synthesis are regulated in a cascade fashion during the lytic cycle (51). This would imply that in addition to unique proteins responsible for differential cell tropism, they probably possess some conserved structural and non-structural proteins responsible for common functions. A great deal of DNA homology has been observed between various herpesviruses, and a number of common antigenic determinants have been reported among human and animal herpesviruses (3,28,32). Based on these previous observations, DNA homology as well as antigenic relationships are also likely to be present among herpesviruses infecting bovid species. Moreover, it has been observed that diagnosis of AHV-1 has been often complicated by the presence of antibodies to other herpesviruses (19). This has prevented adoption of alternate methods of serologic

diagnosis to replace the cumbersome and time consuming virus neutralization assay. In this chapter the degree of antigenic cross reactivity between various herpesviruses is examined. It was the intent of these studies to attempt identification of a protein or proteins that are entirely unique to AHV-1, and not recognized by antisera to any of the other bovid herpesviruses. The identification of such a protein, and monoclonal antibodies against it, would provide the basis for a diagnostic competitive ELISA assay.

## Materials and Methods

### Cells and virus

WC11 strain of AHV-1 obtained from the San Diego Zoo was grown in Vero cells. Bovine herpesvirus 1 (BHV-1;Colorado strain 1 ATCC VR 864) and bovine herpesvirus 2 (BHV-2;NY-1 strain, ATCC VR 845) were grown in primary bovine testicle (BT) cells. Movar 33/63 was grown in Maden-Darby bovine kidney (MDBK) cells, and Suid herpesvirus 1 (SHV-1) was grown in baby hamster kidney (BHK) cells. Cells were infected with BHV-1, BHV-2 and SHV-1 and were labeled with $^{35}S$-methionine (40 $\mu$Ci/ml) by addition of radioisotope at 5 hours post infection in methionine free media and harvested 24 hours later. Cells infected with Movar 33/63 were labeled between 24 and 48 hours post infection.

### Polyclonal antibodies

Antisera was made in rabbits to each of the four herpesviruses. Monolayers of susceptible cells were infected with virus at a multiplicity of infection of 5-10 plaque-forming units (PFU) and incubated at 37°C until CPE was extensive. Cells were harvested and

subjected to two cycles of freeze/thaw to produce a cell lysate. Adult New Zealand White rabbits were initially immunized with 2 ml of cell lysate emulsified in Freund's complete adjuvant. Three subsequent inoculations were given at weekly intervals with 2 ml of the cell lysate emulsified with incomplete Freund's adjuvant, and blood was collected 2 weeks later. Immune rabbit sera (2ml) were absorbed 4 times with $5 \times 10^7$ cells of the type in which the virus was propagated, and 4 times with packed Vero cells and were passed 6 times over a column of Sepharose 4B coupled to a frozen and thawed lysate of noninfected cells of the type in which the virus was propagated. After these absorptions, antisera no longer reacted with the noninfected cells in indirect immunofluorescence assays. Antibody was then purified from sera by chromatography on protein A-agarose, concentrated by dialysis against polyethylene glycol, and protein concentrations determined.

Virus and DNA purification

Virus was pelleted from spent culture media from AHV-1 infected Vero cell cultures. Virus was suspended in TE buffer (10mM Tris chloride, 1mM EDTA) and layered onto a 20-60% continuous sucrose gradient and centrifuged in a SW 28.1 rotor at 15,000 rpm for 50 minutes. The virus band was harvested by penetrating the polycarbonate tube with a needle and withdrawing the band with a syringe. The virus was then diluted in TE buffer and pelleted by centrifugation for 30 minutes at 150,000 g.

The purified virus pellet was suspended in 0.5 ml of TE buffer and protein was digested by the addition of Proteinase K (100$\mu$g/ml) and SDS (0.1%). Digestion was allowed to proceed for 2 hours and the preparation was extracted 2 times alternating with phenol and chloroform. Virus DNA was then precipitated with 100% ethanol and 3M sodium acetate, then pelleted by centrifugation at 15,600 g in an Eppendorf centrifuge. The supernatant was discarded and the pellet was dried under vacuum. The DNA was suspended in TE buffer and stored at 4°C.

DNA Digestion and Southern Blotting

A sample of DNA was added to equal volumes of tracking dye (10% glycerol and bromophenol blue in TE buffer) and electrophoresed in a 1% agarose gel with 0.5% ethidium bromide. The amount of viral DNA was estimated by comparison of intensity of the viral DNA to Hind III digested fragments of lambda DNA. One microgram of DNA was then digested overnight with 2 $\mu$l of restriction endonuclease Pst I, or Hind III. The digested DNA was loaded and electrophoresed on a 1% agarose gel. The gel was denatured in 1.5 M NaCl and 0.5 M NaOH, then neutralized in 1M Tris (pH 8.0) and 1.5 M NaCl. The DNA was then transferred to nitrocellulose and immobilized by baking the nitrocellulose for 2 hours at 80°C under vacuum (34).

Hybridization of Southern Filters

The nitrocellulose containing DNA was wetted with 6X SSC (34) and prehybridized in 6X SSC, 0.5% SDS, 5X Denhardt's solution (34) and 100 $\mu$g/ml of denatured herring sperm DNA in a sealed bag at 56°C for 3

hours. The $^{32}P$ labeled AHV-1 DNA with total counts per minute (CPM) of 2.2 $x10^6$ was added and allowed to hybridize for 12 hours at 56°C. The nitrocellulose was then washed 3 times, for 30 minutes at 56°C, in 2X SSC and 0.1% SDS, dried on Whatman 3MM paper and applied to X-ray film to obtain an autoradiographic image.

DNA Probe

Radiolabeled AHV-1 DNA was made by random primer extension using the Boehringer Manheim Random Primed DNA Labeling Kit. In brief, 1 $\mu$g of AHV-1 DNA was denatured at 95°C for 10 minutes, then cooled on ice. Unlabeled nucleotides dGTP, dTTP, and $^{32}P$ labeled dCTP and dATP, and Klenow enzyme were added to the denatured mixture, which was allowed to incubate for 1 hour. The reaction was then stopped with 2 $\mu$l of .25 M EDTA and non-incorporated nucleotides were removed with an 8 ml desalting column. Fractions were collected from the column, counted on a scintillation counter and the fractions with peak activity were pooled. The radiolabeled probe was added to hybridization solution (5ml formamide, 0.1 ml 20% SDS, 2 ml 20X SSC, 0.5 ml Denhardt's solution, with 100 $\mu$g/ml of denatured herring sperm DNA) and brought to a total volume of 10 ml with sterile autoclaved double deionized water. The probe was denatured by boiling for 5 minutes before hybridization with nitrocellulose.

## Results

### Cross-reactivity of AHV-1 Proteins

Virus-induced proteins (strain WC11) radiolabeled with $^{35}S$-methionine were immunoprecipitated with equal concentrations (35 $\mu$g)

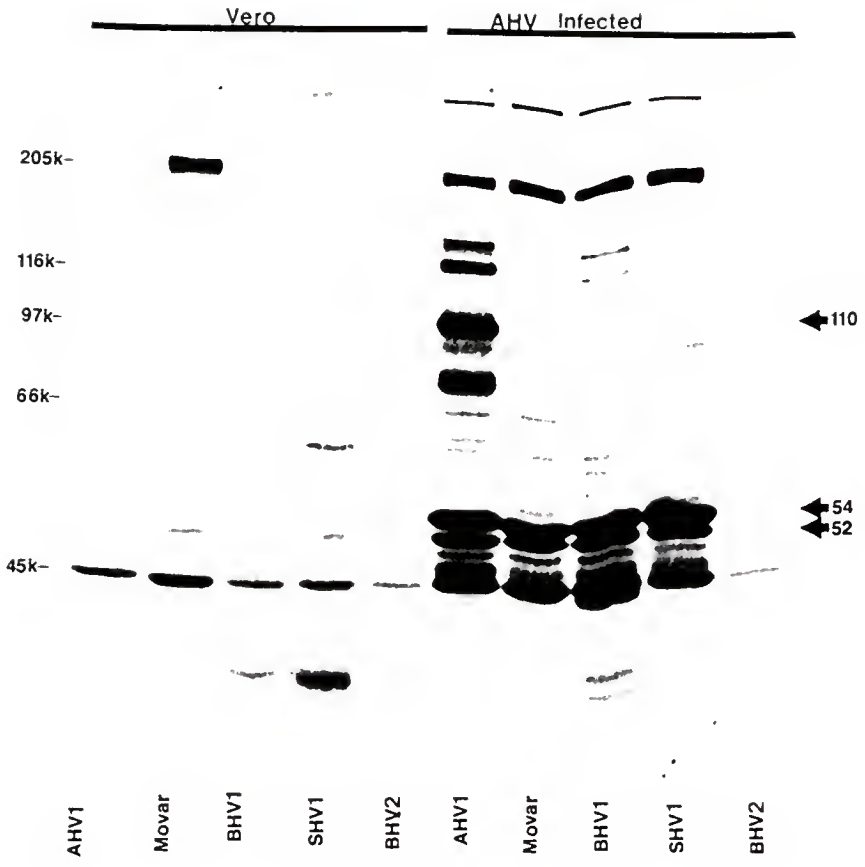


Figure 4.1 - SDS-PAGE analysis of $^{35}$S-methionine labeled proteins immunoprecipitated from uninfected (Vero) and AHV-1 infected (AHV Infected) Vero cells with equal concentrations of rabbit polyclonal antibodies to AHV-1, Movar, BHV1, SHV1 and BHV2. Arrows to the right indicate proteins immunoprecipitated from infected cells and their apparent molecular weights (x1000).

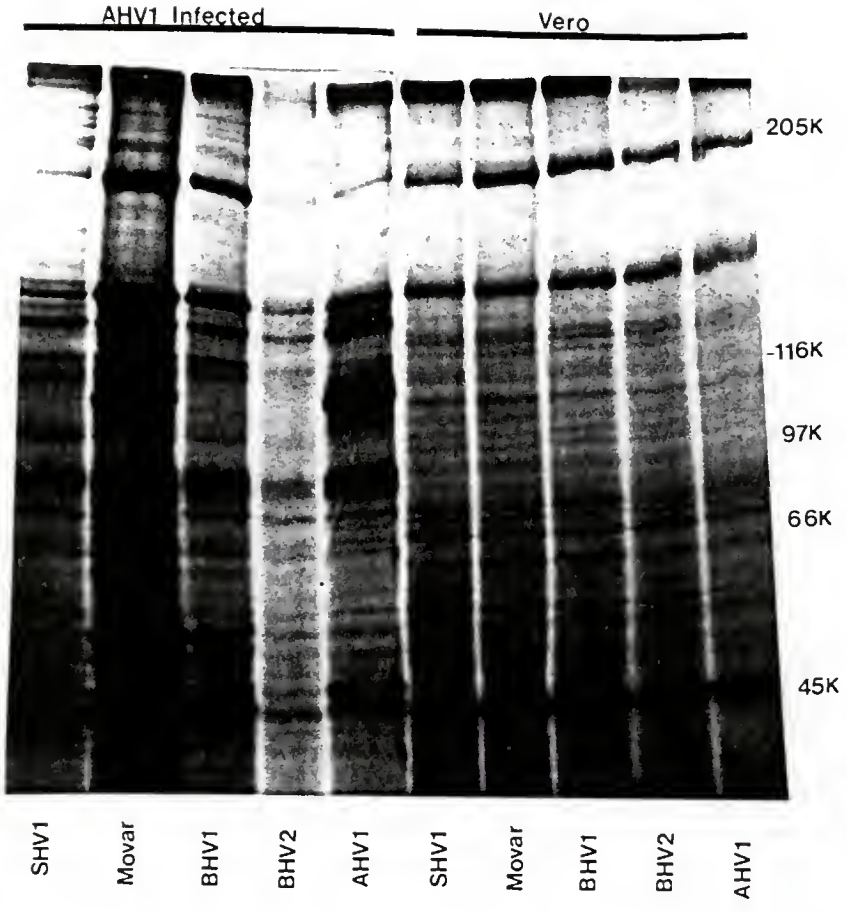


Figure 4.2 - SDS-PAGE analysis of $^{35}$S-methionine labeled proteins immunoprecipitated from uninfected (Vero), and AHV-1 infected (AHV Infected) Vero cells with unequal concentrations of rabbit polyclonal antibodies to SHV1, Movar, BHV1, BHV2 and AHV1. Numbers to the right indicate position of molecular weight standards and their apparent molecular weights (x1000).

of purified rabbit polyclonal antibody against AHV-1, BHV-1, BHV-2, Movar, and SHV-1. Some antigenic cross reactivity was seen in this analysis with most AHV-1 proteins (Figure 4.1). This cross-reactivity was especially notable for proteins of 52 kDa and 54 kDa. There was however, also a notable lack of reactivity of a 110 kDa protein with any antibodies other than those made to AHV-1 itself. In fact this protein appeared completely unique to AHV-1.

If this experiment was repeated using equal volumes of antibody, a rather different pattern was seen (Figure 4.2). The total amounts of each polyclonal antibody used was 35 $\mu$g anti-AHV-1, 48 $\mu$g anti-SHV-1, 55 $\mu$g anti-BHV-1, 115 $\mu$g anti-BHV-2, and 175 $\mu$g anti-Movar. Cross-reactivity now appeared extensive. The 110 kDa protein that appeared unique when equalized concentrations of antibody are used, was precipitated by the antibodies raised to Movar when these were present in higher concentrations.

DNA Hybridization

DNA was isolated from AHV-1, BHV-2 and Movar. This DNA was digested by restriction endonucleases Eco RI and Hind III, and run on a 1% agarose gel. The DNA was then transferred to nitrocellulose and blotted with the $^{32}P$ labeled AHV-1 DNA. Hybridization was extensive in lanes containing AHV-1 DNA (Figure 4.3). Hybridization was also seen with one major band and several (at least six) minor bands ranging in size from approximately 4 to 9 kilobases in both the Eco RI and Hind III digests of Movar 33/63. No hybridization was noted between the AHV-1 probe and digested BHV-2 DNA even after long

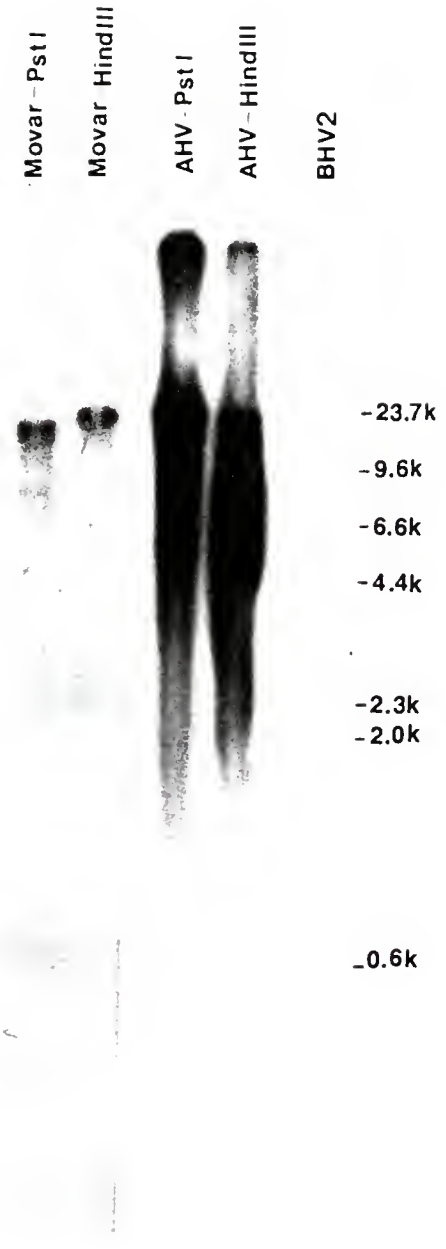


Figure 4.3 - Southern blot analysis of DNA isolated from WC11 strain of AHV-1, BHV-2, and Movar digested with restriction endonucleases Pst I and Hind III and hybridized with WC11 AHV-1 labeled with $^{32}P$, by random primer extension. Numbers to the right indicate positions of lambda-Hind III fragment standards, and their sizes in kilobases.

exposure of the probed nitrocellulose to radiographic film (Figure 4.3).

Unique epitopes

Cross immunoprecipitation experiments did not identify a single protein that could be assumed to be unique to AHV-1. We therefore sought to identify at least individual epitopes unique to AHV-1. In order to do this, susceptible cell lines were infected with BHV-1, BHV-2, SHV-1 and Movar and labeled with $^{35}$S-methionine as described above. Lysates were made of the infected cells, and samples were immunoprecipitated with polyclonal rabbit antibodies to the respective viruses, as well as to AHV-1. Samples of lysate were also immunoprecipitated with 2 monoclonal antibodies. Since a neutralization assay is currently the only method capable of distinguishing animals infected with AHV-1 from those carrying other herpesviruses, we reasoned that antibodies capable of neutralizing infectivity might be the most likely to recognize unique AHV-1 epitopes. One antibody, 12B5, was selected because of its ability to neutralize virus in vitro. This antibody recognizes the AHV-1 115 kDa and associated proteins. The second antibody selected was 31B3. This antibody does not have neutralizing activity at similar antibody concentrations, but immunoprecipitates the same protein complex as monoclonal 12B5. It was found that monoclonal antibodies 12B5 and 31B3 did not specifically react with $^{35}$S-methionine labeled proteins of BHV-1, BHV-2, Movar or SHV-1 (Figure 4.4, 4.5). These data suggested that the 2 epitopes recognized by the monoclonal antibodies were likely to be unique to AHV-1.

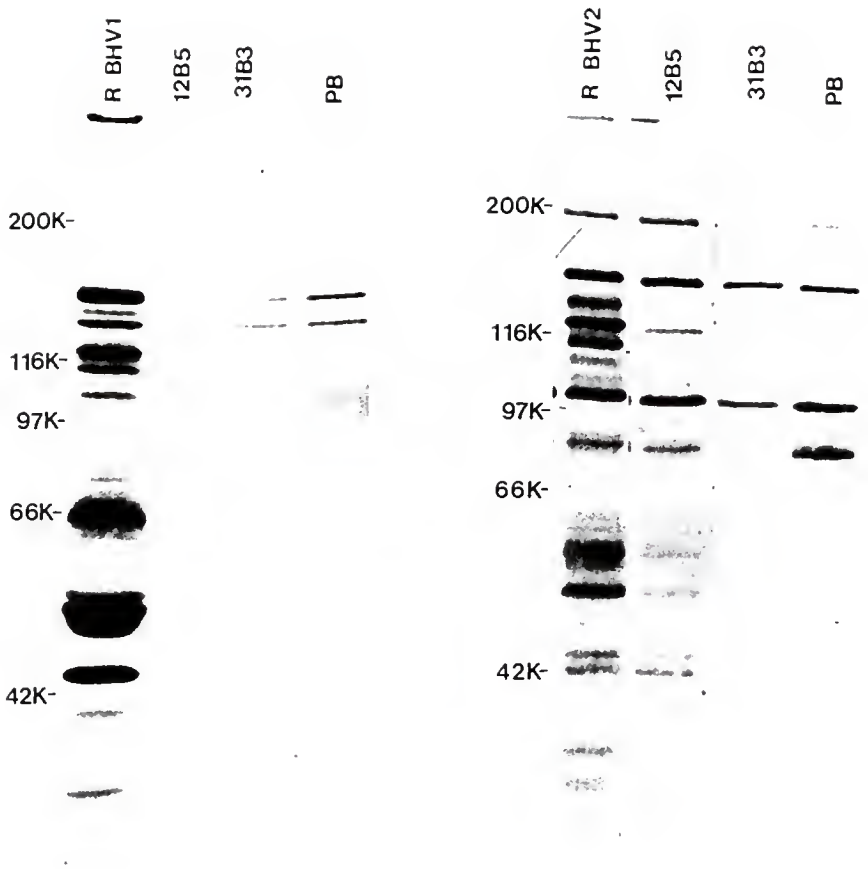


Figure 4.4 - SDS-PAGE analysis of $^{35}$S-methionine labeled BHV-1 proteins (left 4 lanes), and BHV-2 proteins (right 4 lanes) immunoprecipitated with their respective polyclonal antibodies (anti-BHV-1 or anti-BHV-2), monoclonal antibodies 12B5 and 31B3 and non-immune antibodies (PB). Numbers to the left indicate positions of molecular weight standards and their apparent molecular weights (x1000).

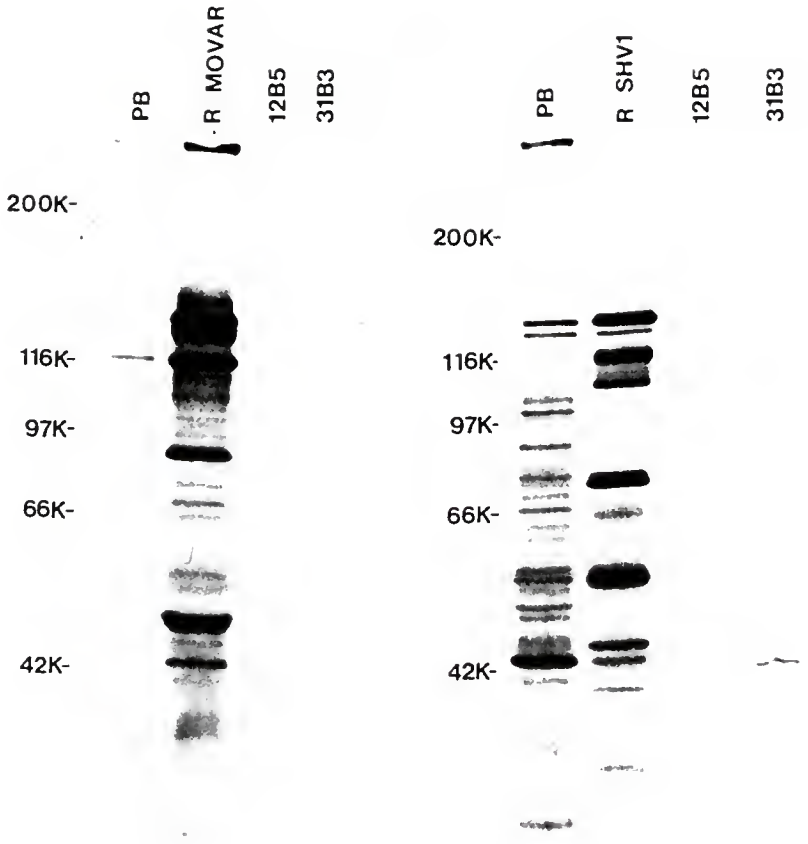


Figure 4.5 - SDS-PAGE analysis of $^{35}$S-methionine labeled Movar proteins (left 4 lanes), and SHV-1 proteins (right 4 lanes) immunoprecipitated with their respective polyclonal antibodies (anti-Movar or anti-SHV-1), monoclonal antibodies 12B5 and 31B3 and non-immune antibodies (PB). Numbers to the left indicate positions of molecular weight standards and their apparent molecular weights (x1000).

## Discussion

Antigenic cross-reactivity between AHV-1 and the other herpesviruses tested were expected because of earlier work of others (3,28). Immunoprecipitation of AHV-1 proteins with polyclonal antibodies to other bovid herpesviruses suggested that AHV-1 was more closely related to Movar than it was to BHV-1, BHV-2 or SHV-1. To further test the degree of relationship between these two viruses, the degree of DNA homology as evaluated by cross-hybridization of DNA from AHV-1 and Movar was determined. Although not extensive, a moderate degree of homology was shown to exist at the DNA level. This finding further substantiated a closer relationship between these two viruses and was consistent with data generated by ourselves and others that indicated antigenic cross reactivity between AHV-1 and Movar (43,57).

Although the various herpesviruses are undoubtedly antigenically related to some degree, the fact that the virus neutralization assay was discriminating suggested that proteins with neutralizing epitopes might be wholly or in part unique to AHV-1. It was our intention to identify a protein that was entirely unique to AHV-1, that might provide the basis of a diagnostic assay. Monoclonal antibodies or polyclonal monospecific antibodies to this protein might then be used in the development of a competitive ELISA. Alternatively, cloning of the gene encoding this protein, and its subsequent expression, might allow its purification in relatively large amounts. The purified protein could then be used as antigen in a direct or indirect ELISA. This approach could provide a very specific and rapid means of diagnosis.

An entirely unique protein could not be definitively identified by immunoprecipitation of AHV-1 proteins with polyclonal antibodies to other bovid herpesviruses. An alternate approach was thus pursued. Identification of monoclonal antibodies that reacted with individual unique epitopes on AHV-1 that were not shared with the other viruses provided reagents that could be used in the development of a prototype diagnostic assay for AHV-1 antibodies. These monoclonal reagents provide the basis for a prototype of an improved diagnostic assay described in Chapter 5.

# CHAPTER 5

# PROTOTYPE DIAGNOSTIC ELISA

## Introduction

The following chapter describes a preliminary assessment of a prototype diagnostic assay for infection with AHV-1. This assay will be based on a competitive ELISA, which tests the ability of sera from animals exposed to AHV-1 to compete for the binding of a monoclonal antibody to AHV-1 antigen. If an assay of this type proves to be sensitive and specific, it will provide a useful tool for rapid, safe and relatively inexpensive diagnosis of AHV-1 infection.

## Materials and Methods

### ELISA Reagents

The antigen in this assay was composed of protein from WC11 infected Vero cells, and was used at concentrations equal to that used in the ELISA described previously for the screening of hybridomas. Plates were coated with antigen in bicarbonate/carbonate buffer pH 9.6 and washed 3 times in wash buffer containing 0.05% Tween 20, and 0.85% NaCl. Samples being tested for the presence of antibodies against AHV-1 were added to plates for one hour at 37°C, and followed by addition of monoclonal antibody 12B5 at a concentration of 0.1 $\mu$g per well. The mixture was allowed to incubate for an additional 2 hours, washed 3 times with wash buffer and incubated for 1 hour at 37°C with goat anti-mouse antibody conjugated with horse radish peroxidase

and washed again. The amount of anti-mouse antibody bound was visualized by a change in the color of the substrate ortho-phenylenediamine (Sigma). The reaction was stopped with 2M $H_2SO_4$, and the absorbance at 492 nm measured with an ELISA reader. The ability of the serum samples to compete with the monoclonal antibody for binding to the antigen was expressed as percent of control values (see below).

The optimal concentration of monoclonal antibody to be used in the assay was determined in tests using a serum sample from a bison with clinical disease and known to contain antibodies to AHV-1, and a known negative serum sample. Concentrations of monoclonal antibody ranging from 0.1$\mu$g to 100 $\mu$g per well were examined. Results were expressed as a percentage of the optical density of a well that had been reacted with the monoclonal antibody, but no competing serum sample. That is to say that the value is expressed as a percentage of the maximum possible optical density in the absence of competing serum antibodies.

Serum samples

Polyclonal rabbit sera made against AHV-1, BHV-1, BHV-2, Movar, and SHV-1, were tested for their ability to compete with the monoclonal antibody 12B5 for binding with AHV-1 antigen. Also tested were 3 bovid serum samples (2 wildebeest and 1 bison) known to be positive and 4 bovid sera (1 wildebeest, 1 domestic cow, 1 moose and 1 domestic goat) known to be negative by virus neutralization. In addition a total of 8 serum samples from sheep associated with a

recent outbreak out malignant catarrhal fever in Alaskan moose were tested. Five samples were known to be positive and two samples were known to be negative by virus neutralization. One sheep sample was considered a borderline positive by the neutralization assay. Thirteen serum samples obtained from a sentinel cattle herd belonging to the University of Florida, and presumed to be negative, were also assayed.

## Results

### Titration of 12B5

The optimum concentration of monoclonal antibody 12B5 for use in the competitive ELISA was found to be 0.1 $\mu$g per well. This concentration produced the maximum difference in reactivity between sera with and without antibodies to AHV-1 (Figure 5.1).

### Rabbit antibodies

Equal concentrations of purified polyclonal antibodies against AHV-1, BHV-1, BHV-2, Movar and SHV-1 were tested in the ELISA to determine their ability to compete with the monoclonal antibody 12B5 for binding to AHV-1 antigen. In this experiment results were expressed as a percentage of the optical density in a well containing equal concentrations of non-immune rabbit antibody. At 20 $\mu$g of antibody there was a clear distinction between the ability of AHV-1 antibody to compete for antigen versus that of the other polyclonal antibodies. Figure 5.2 illustrates that when between 0.5 $\mu$g and 20 $\mu$g of each polyclonal antibody was used in this assay, the AHV-1 antibody was better able to compete with binding of antibody than any of the

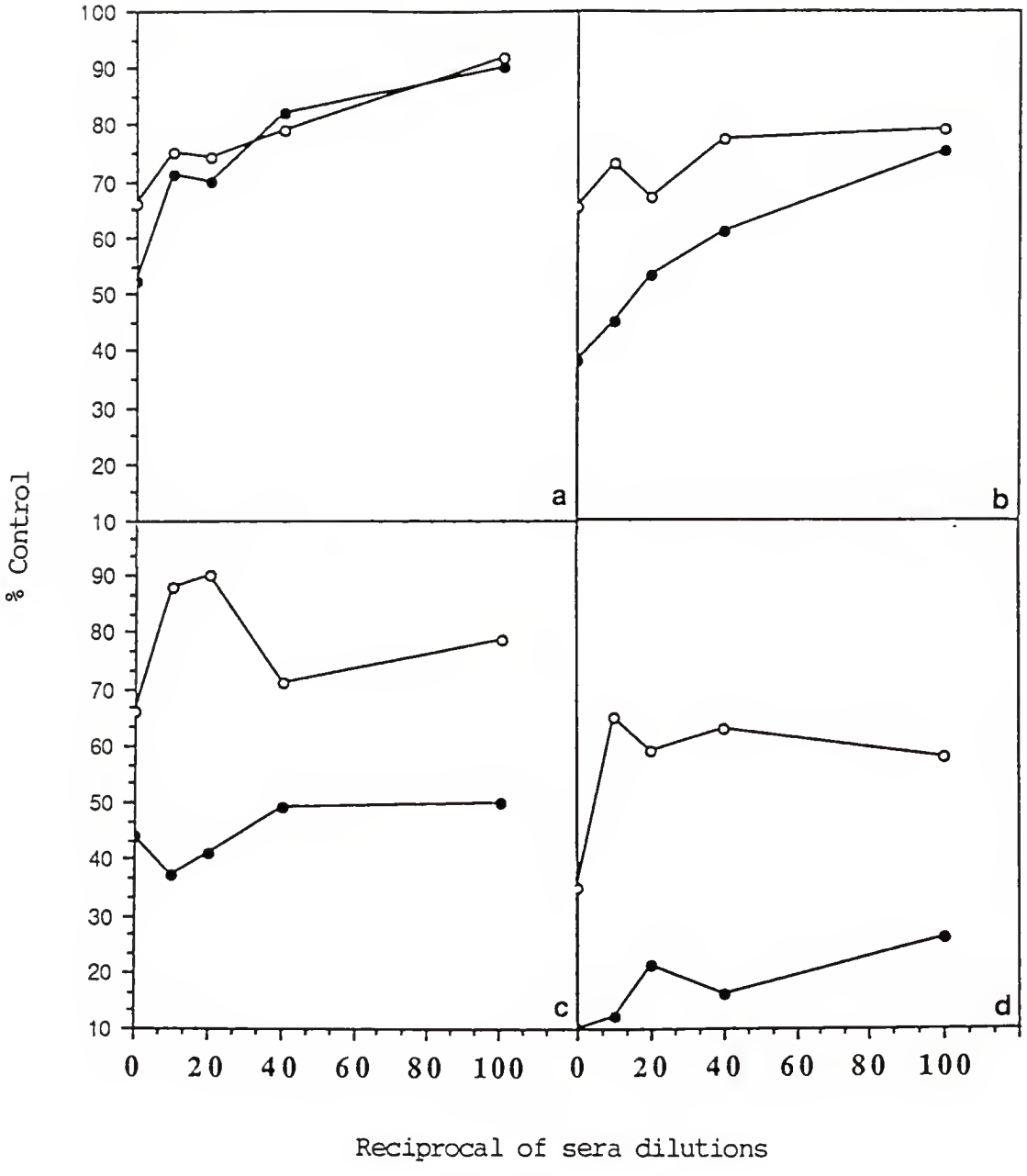


Figure 5.1 - Titration of monoclonal antibody 12B5 for use in a competitive ELISA. Percent control is calculated as the optical density of wells containing serum and monoclonal antibody 12B5 divided by the optical density of wells containing monoclonal antibody only x100. ●- Positive bison serum. O-Negative goat serum. Monoclonal antibody 12B5 used at concentrations of a) 100 μg/ml b) 10 μg/ml c) 1 μg/ml d) 0.1 μg/ml.

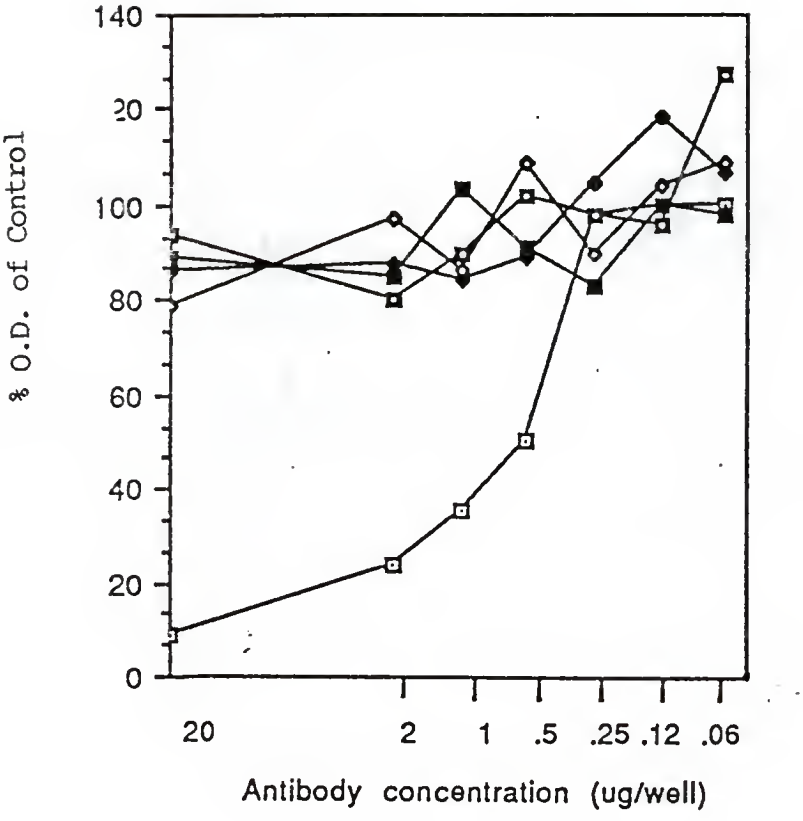


Figure 5.2 - Analysis of the ability of rabbit polyclonal antibodies raised against various bovine herpesviruses to reduce binding of monoclonal antibody 12B5 to AHV-1 infected cell lysate in a competitive ELISA. Percent control is calculated as the optical density of wells containing the polyclonal antibody sample divided by the optical density of wells containing equal concentrations of non-immune rabbit antibodies x 100. □ - rabbit anti-AHV-1, ◆ - rabbit anti-Movar, ◘ - rabbit anti-BHV-1, ◇ - rabbit anti-BHV-2, ■ - rabbit anti-SHV-1.

other polyclonal antibodies. When the anti AHV-1 antibody concentration dropped below 0.5 $\mu$g, it no longer competed with the monoclonal antibodies for binding to antigen.

Bovid sera

The bovid serum samples were diluted 1:10 and tested for their ability to compete with monoclonal antibody for binding to antigen. The data illustrating the ability of the serum samples to compete are expressed as percent inhibition of binding. This was calculated by the formula: % inhibition of binding = a-b/a, where a = the optical density in wells containing negative sera and monoclonal antibody 12B5, and b = the optical density in wells containing test sera and monoclonal antibody 12B5. The 3 known AHV-1 positive serum samples at dilutions of 1:10 were able to each compete with monoclonal antibody for binding to the antigen. When these 3 samples were compared with known negative serum samples, they were able to reduce the binding of monoclonal antibody from 35 to 50% (Figure 5.3). The 3 known negative serum samples reduced the ability of the monoclonal antibody to bind AHV-1 antigen by less than 1% (Figure 5.3). The serum samples from the UF sentinel herd reduced the binding from 0 to a maximum of 25% (Figure 5.3). Four of the known positive sheep samples reduced binding from 22 to 30%, and all of the known negative sheep samples reduced binding less than 6%. One sheep sample that was determined to be borderline-positive by a virus neutralization conducted by the San Diego Zoo, was the median value of the sheep samples in its ability to

reduce binding to AHV-1 antigen. One known positive sheep serum sample reduced binding only 8% (Figure 5.4).

## Discussion

Although very preliminary, the results thus far generated with the prototype competitive ELISA strongly suggested that it has promise for identification of animals infected with AHV-1. The assay was clearly capable as differentiating rabbit polyclonal antibodies raised against AHV-1 and the other bovid herpesviruses. In addition, serum from bovids testing positive with the virus neutralization assay were better able to compete with monoclonal antibody 12B5 for binding with antigen than were 4 serum samples shown to be negative by the same test. When the 13 cattle from the UF sentinel herd were tested, the ability to compete for binding with monoclonal 12B5 was more variable, but no samples were able to compete more than 25%.

It has been demonstrated that serum from sheep associated with outbreaks of malignant catarrhal fever in cattle are capable of neutralizing AHV-1 virus in plaque reduction protocols (22). The interpretation of these findings was that the sheep associated form of malignant catarrhal fever is caused by an as yet unidentified virus which is very similar to AHV-1. Our data support this interpretation.

The total number of animals tested in this assay is to date very small, and must be expanded before statistical analysis of its predictive value can be determined. If after further analysis, the monoclonal 12B5 and the infected cell lysate as antigen does not allow specific diagnosis, several alternative approaches might be pursued.

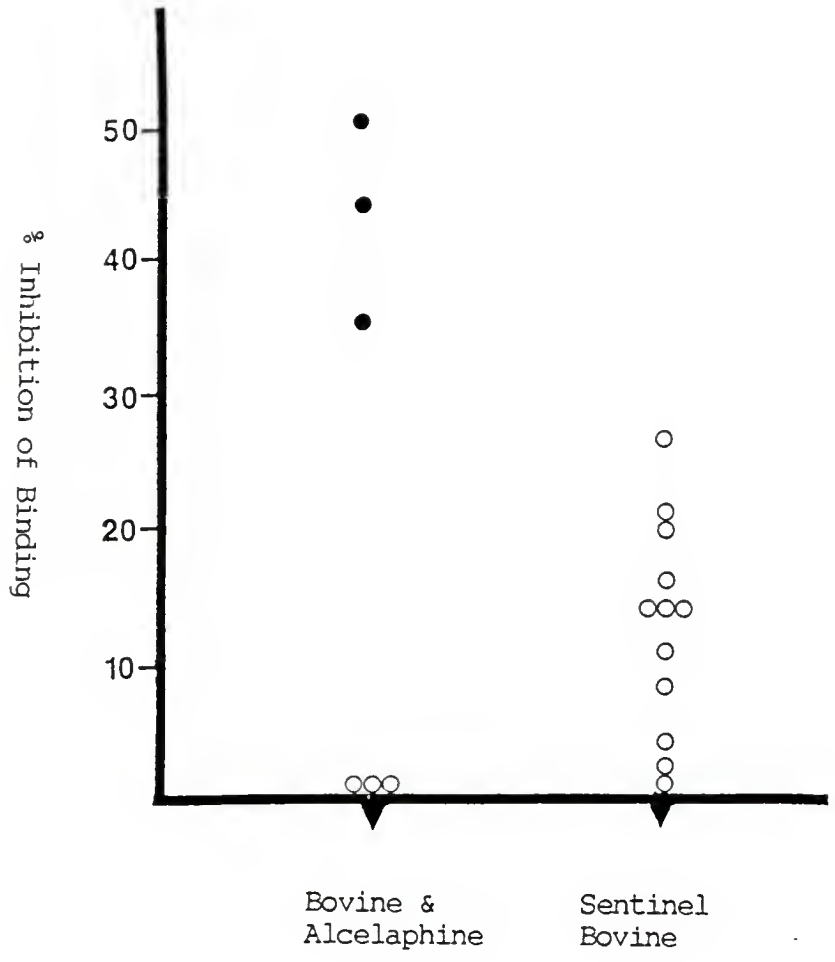


Figure 5.3 - Ability of serum to inhibit binding of monoclonal antibody 12B5 to AHV-1 infected cell antigen. Percent inhibition of binding = a-b/a; where a = Optical density in wells containing monoclonal antibody and known negative serum, b = Optical density in wells containing monoclonal antibody and test sera. ● - Samples known to be positive by virus neutralization assay. O - Bovine and Alcelaphine serum samples known to be negative by virus neutralization and Bovine samples from sentinel herds presumed to be negative.

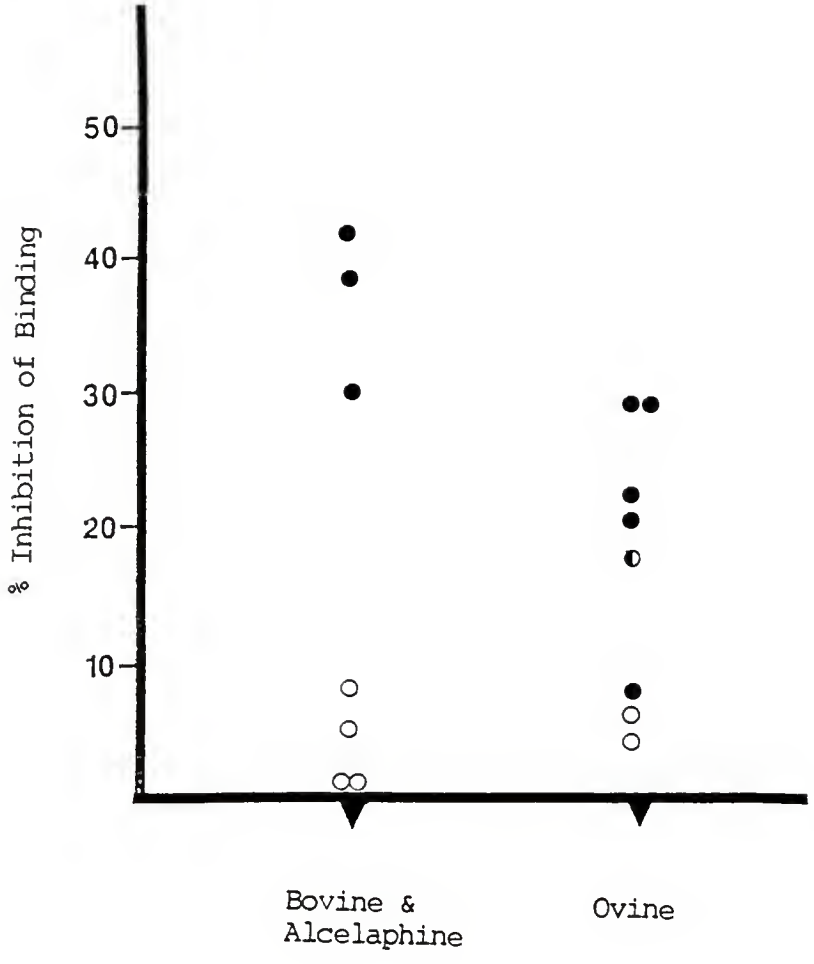


Figure 5.4 - Ability of Alcelaphine, Bovine, and Ovine serum to inhibit binding of monoclonal antibody 12B5 to AHV-1 infected cell antigen. Percent inhibition of binding = a-b/a; where a = Optical density in wells containing monoclonal antibody and known negative serum, b = Optical density in wells containing monoclonal antibody and test sera. ● - Samples known to be positive by virus neutralization. O- Samples known to be negative by neutralization. ◐ - Borderline result in virus neutralization assay.

An alternate monoclonal antibody to the 115 kDa or some other protein may yield better results. The fact that antibody 12B5 does neutralize in vitro implies that the epitope recognized by the antibody is unique to AHV-1. If an alternate antibody were to be tested in this assay, it would seem logical to select another capable of neutralizing AHV-1 in vitro. If the assay does prove to have a good predictive value, production of antigen on a large scale might be attempted by the cloning of the gene gp115. Cloning of the gene into a plasmid expression vector might suffice to produce large amounts of a fusion protein for use as an antigen. One potential problem with this technique is that the bacterially expressed protein will not be glycosylated and therefore may not be recognized by the monoclonal antibodies that we have available. This would necessitate one of two additional approaches. We could use a mammalian vector system such as vaccinia virus to produce a protein that was processed more like the native molecule, but a better approach would probably be to use our fusion protein to immunize mice to produce additional monoclonal antibodies. Sera would then need to be tested for their ability to compete these new monoclonal antibodies from the purified fusion protein. One or more of these approaches should lead to the refinement of this assay as a tool for the identification of carrier animals infected with AHV-1, and possibly the sheep-associated virus.

CHAPTER 6

# THE gp115 SURFACE PROTEIN

## Introduction

Several hybridoma antibodies made against AHV-1 were found to react with a virus induced complex that consisted of 5 proteins that could be labeled with $^{35}$S-methionine. One of the antibodies was also capable of neutralizing virus in plaque reduction assays. The neutralizing antibody recognized the same epitope, or one very close to it, as sera taken from naturally infected animals inhibited binding of the monoclonal antibody to AHV-1 antigen (see Chapter 4). The complex thus may include a molecule(s) of significance to both diagnosis and prevention of the disease. Two hybridoma antibodies that recognized the complex, 12B5 which neutralized infectivity and 31B3 which did not, were repeatedly cloned and used to investigate the nature and origin of the protein complex.

## Materials and methods

### Gel electrophoresis

SDS-PAGE electrophoresis was performed as described previously, except that 2-mercaptoethanol was omitted from the sample buffer when gels were to be run under non-reducing conditions.

Pulse-chase experiments

Monolayers of BEL cells were infected with approximately 5 PFU per cell of strain 1982 of AHV-1 and infection was allowed to proceed for 36 hours. At 36 hours post infection the cells were pulsed with 100 $\mu$Ci/ml of $^{35}$S-methionine in methionine free media for 15 minutes and chased with cold methionine for periods of 15, 30, 45, 60, 120 minutes or 24 hours. To achieve chase of radiolabel the media containing the $^{35}$S-methionine was removed and replaced with fresh media containing 100 $\mu$g of cycloheximide and 100X the normal concentration of methionine. At the end of each chase period cells were scraped from the flask with a rubber policeman into ice cold PBS, pelleted and stored at -20°C.

N-glycanase digestion

Strain 1982 infected BEL cells were labeled with $^{35}$S-methionine, and lysed as described previously. Two samples were immuno-precipitated with monoclonal antibody 12B5 and protein A-agarose. The protein A-agarose beads with bound 12B5 antibody and radiolabeled protein were washed 3 times with RIPA buffer and suspended in 33 $\mu$l of .2M phosphate buffer pH 8.6 and 0.5% SDS and 0.7% 2-mercaptoethanol. The samples were then boiled for 3 minutes, and to each was added 20 $\mu$l 0.67 M phosphate buffer pH 8.6, 10 $\mu$l 0.1 M phenanthroline, 16.7 $\mu$l 7.5% NP-40 and 10 $\mu$l of distilled water. The mixture was vortexed. To one sample was added 2.5 U of N-glycanase in 10 $\mu$l glycerol, and to the other an equivalent volume of glycerol without enzyme. The mixtures were mixed with an equal volume of sample buffer and analyzed by SDS-PAGE.

## Results

### Reducing vs. Non-Reducing Conditions

When the complex immunoprecipitated by antibody 12B5 was analyzed by SDS-PAGE under reducing conditions 5 proteins were seen. The molecular weights of these proteins were 115,000, 110,000, 105,000, 78,000, and 48,000. When the same complex was analyzed under non-reducing conditions, only 3 proteins were seen. The proteins of 78 kDa and 48 kDa were not detected, and the 115 kDa protein was present in very large amounts (Figure 6.1).

If the monoclonal antibody 12B5 was used to immunoprecipitate a lysate of $^{125}I$ labeled 1982 virions, only one protein of 115 kDa was identified under non-reducing conditions. Under reducing conditions it was replaced by two smaller proteins of 78 kDa and 48kDa (Figure 6.2). This suggested to us that the mature protein found on the virion was a heterodimer composed of 2 fragments of 78 kDa and 48 kDa linked by disulfide bonds. We supposed that the molecules of 110 kDa and 115 kDa seen in cells labeled with $^{35}S$-methionine were processing intermediates. To elucidate the relationships of the proteins further we carried out pulse chase experiments as described above. Proteins were immunoprecipitated from each pulse and chase period and analyzed under reducing or non-reducing conditions. Irrespective of the presence or absence of 2-mercaptoethanol, only one protein of 110 kDa was specifically immunoprecipitated by 12B5 from the 15-minute pulse period (Figure 6.3 and 6.4). We presumed this to be the primary translation product. During the 15-minute chase period two proteins were visible under both reducing and non-reducing conditions, the

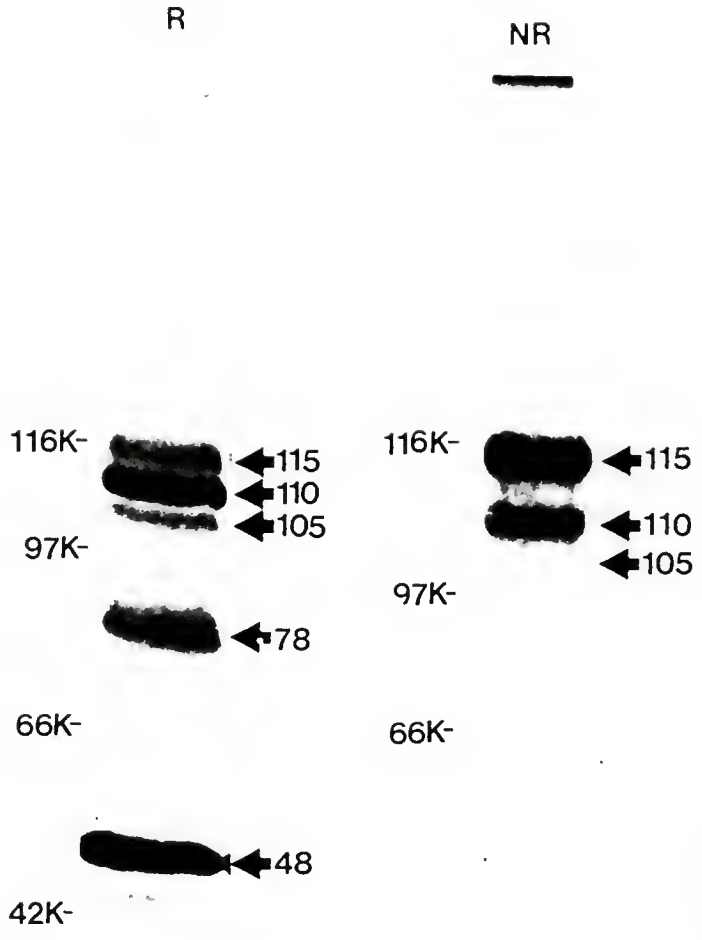


Figure 6.1 - SDS-PAGE analysis of $^{35}$S-methionine labeled proteins immunoprecipitated from cells infected with monoclonal antibody 12B5, and electrophoresed under reducing (R) and non-reducing (NR) conditions. Arrows to the right indicate proteins immunoprecipitated from infected cells and their apparent molecular weights (x1000).

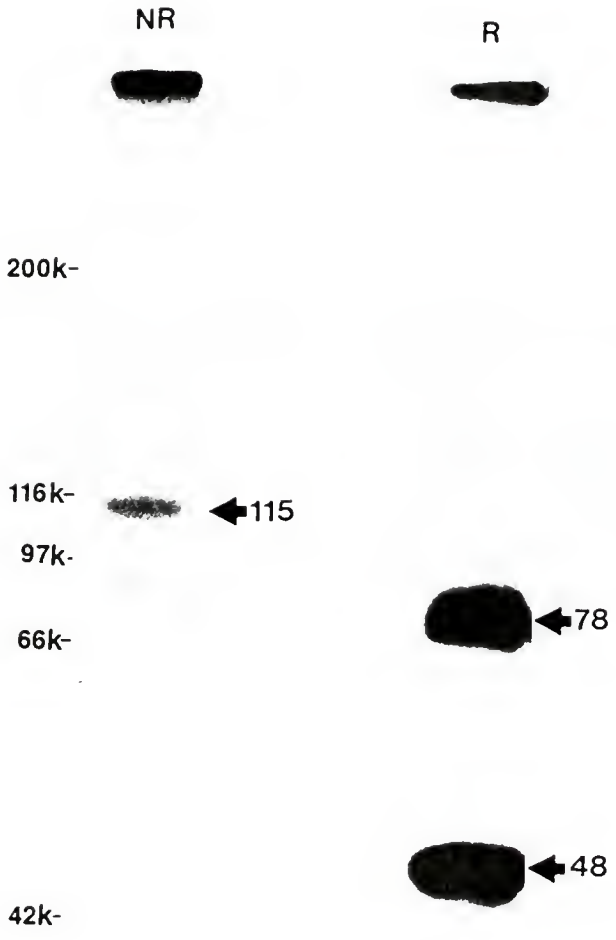


Figure 6.2 - SDS-PAGE analysis of proteins immunoprecipitated from $^{125}I$ labeled 1982 virions immunoprecipitated with monoclonal antibody 12B5 and electrophoresed under reducing (R), and non-reducing (NR) conditions. Arrows to the right indicate proteins immunoprecipitated from virion lysate and their apparent molecular weights (x1000).

heavily labeled 110 kDa protein and a lighter band representing a protein at 105 kDa. After a 30-minute chase period an additional molecule of 115 kDa could be seen. The 45-minute chase period was the first time interval at which the protein patterns were clearly different under reducing and non-reducing conditions. During this chase period, the 115 kDa protein seen under non-reducing conditions was a rather heavy band.In contrast the 115 kDa protein was barely noticeable when run under reducing conditions. Instead, the 115 kDa protein appeared to be replaced by 2 proteins, one at 78 kDa and one at 48 kDa. This pattern remained essentially unchanged throughout the remainder of the chase periods examined, except that the 115 kDa protein gradually became more prominent with time under non-reducing conditions (Figure 6.3) and the 78 kDa and 48 kDa proteins likewise increased in intensity under reducing conditions.

Identity of 115 kDa protein

The $^{35}$S-methionine labeled proteins immunoprecipitated by 12B5 from infected cells migrated slightly faster under non-reducing conditions than under reducing conditions. Molecules of 115, 110, and 105 kDa were seen in reducing gels. These we assumed to be the same proteins as those that migrated with apparent molecular weights of slightly less than 115, 110 and 105 kDa. This made it difficult to be absolutely certain of the identity of the 2 groups. We were most interested in substantiating what our earlier data had suggested; namely that the 115 kDa molecule was actually a heterodimer composed of the 78 kDa and 48 kDa molecules. In order to test this hypothesis, the following experiment was performed. Bovine embryonic lung cells

were infected and labeled with $^{35}$S-methionine and immunoprecipitated with 12B5. The immunoprecipitate was then analyzed by SDS-PAGE. The gel was fixed for 15 minutes with a solution of 7% acetic acid and 7% methanol. A 3 mm punch was then used to cut out a series of acrylamide plugs starting from the top of the gel to below the 58 kDa molecular weight marker. All plugs were then placed in scintillation fluid and counted in a scintillation counter. Two major peaks of radioactivity were seen in regions of the gel to which the proteins of 115 kDa and 110 kDa were expected to migrate (Figure 6.5). The regions of the gel corresponding with location of these peaks were excised and protein electroeluted from them at 100 V overnight at 4°C. A sample containing a total of 8700 CPM of radioactivity from each protein fraction was loaded onto a 9% gel in an equal volume of reducing sample buffer and electrophoresed. Exposure of the original gel confirmed that the 115 kDa and the 110 kDa bands had been excised. Exposure of the second gel indicated that 2 proteins of 78 kDa and 48 kDa originated from the 115 kDa protein (Figure 6.6). The 110 kDa protein apparently remained intact except that a new molecule was seen under reducing conditions with molecular weight of approximately 160 kDa and on overexposure of the gel a faint band of 48 kDa was seen. The 115 kDa protein was almost entirely reduced to 48 kDa and 78 kDa molecules, although a small amount of protein could be seen as a light band running with a mobility of 155 kDa (Figure 6.6).

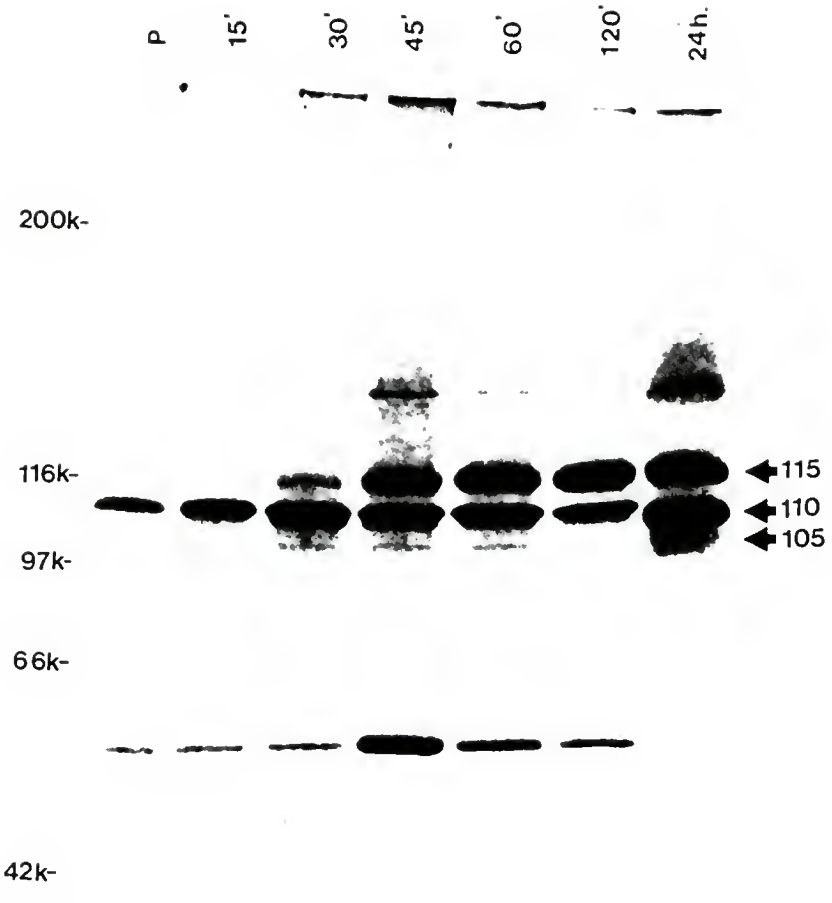


Figure 6.3 - SDS-PAGE analysis of proteins immunoprecipitated by monoclonal antibody 12B5, and electrophoresed under non-reducing conditions. Lanes show infected cells (P) pulsed with $^{35}$S-methionine for 15 minutes at 24 hours post infection, and chased for 15 minutes at 24 hours post infection followed by chase for 15 minutes, 30 minutes, 45 minutes, 60 minutes, 120 minutes, and 24 hours, respectively, in media containing cycloheximide and 100X cold methionine. Arrows indicate proteins immunoprecipitated with monoclonal antibody 12B5.

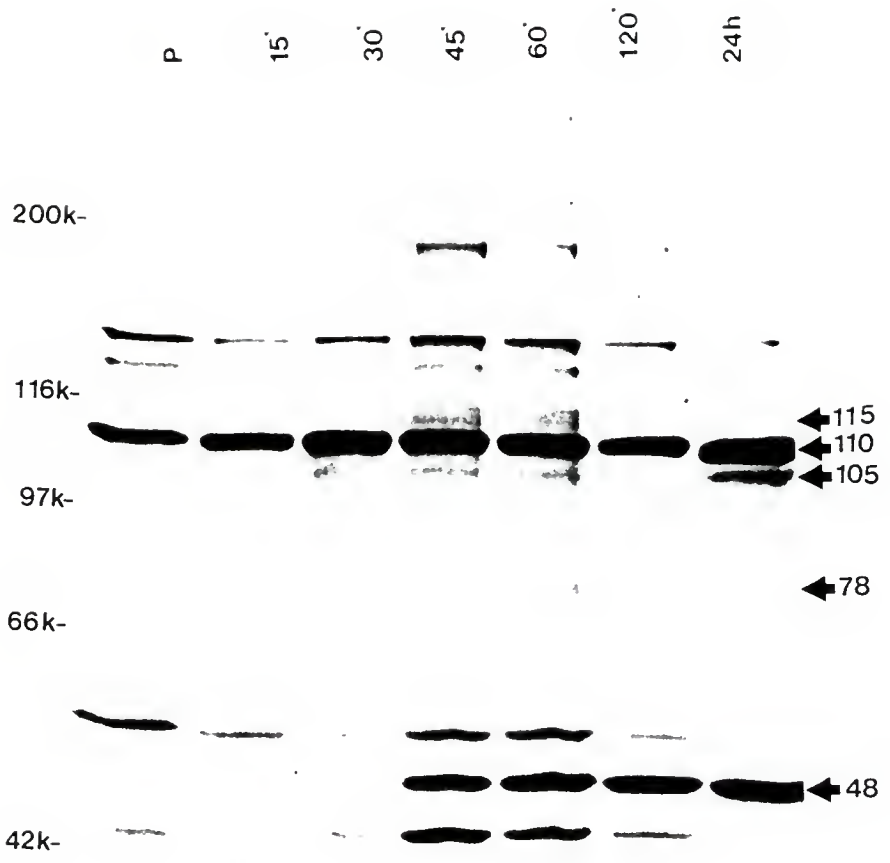


Figure 6.4 - SDS-PAGE analysis of proteins immunoprecipitated by monoclonal antibody 12B5, and electrophoresed under reducing conditions. Lanes show infected cells (P) pulsed with $^{35}$S-methionine for 15 minutes at 24 hours post infection, and chased for 15 minutes at 24 hours post infection followed by chase for 15 minutes, 30 minutes, 45 minutes, 60 minutes, 120 minutes, and 24 hours, respectively, in media containing cycloheximide and 100X cold methionine. Arrows indicate the positions of the proteins specifically immunoprecipitated with 12B5.

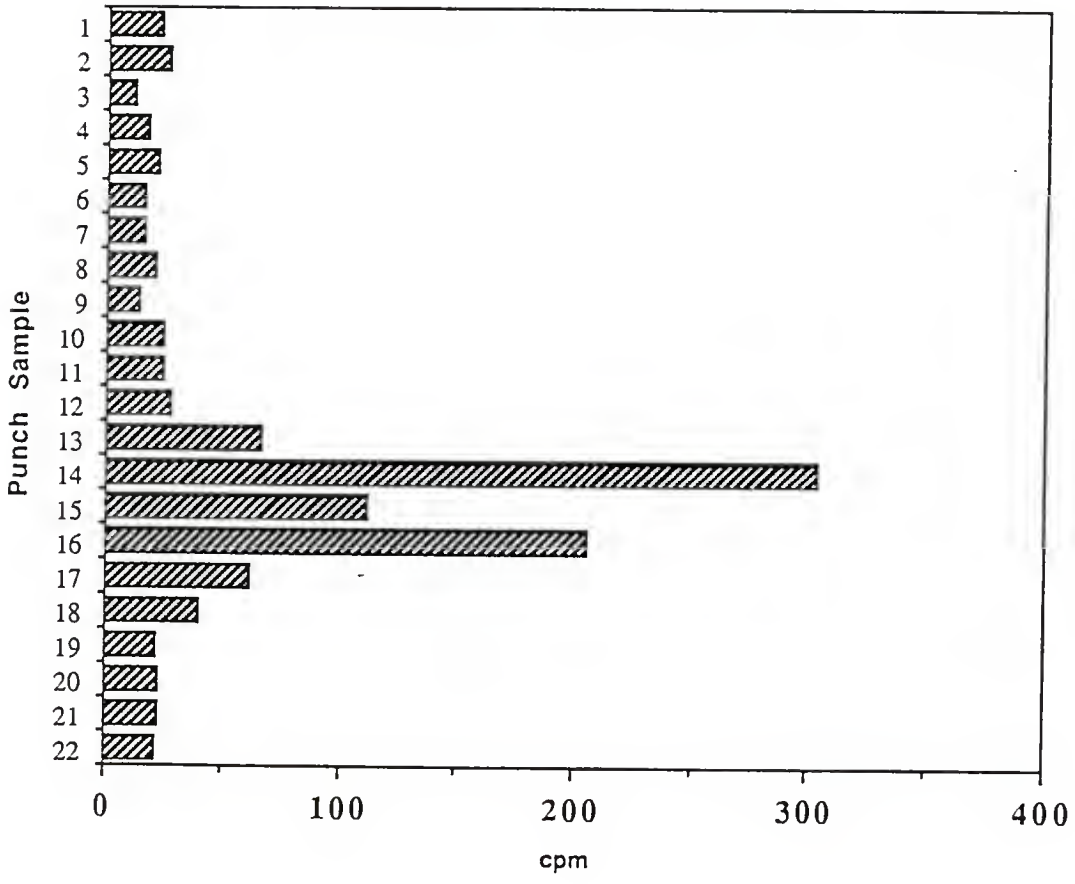


Figure 6.5 - Histogram indicating counts per minutes in 3 mm punch samples that were used to identify the location of $^{35}$S-methionine labeled proteins of AHV-1 immunoprecipitated by monoclonal antibody 12B5 and electrophoresed in a 9% polyacrylamide gel under non-reducing conditions.

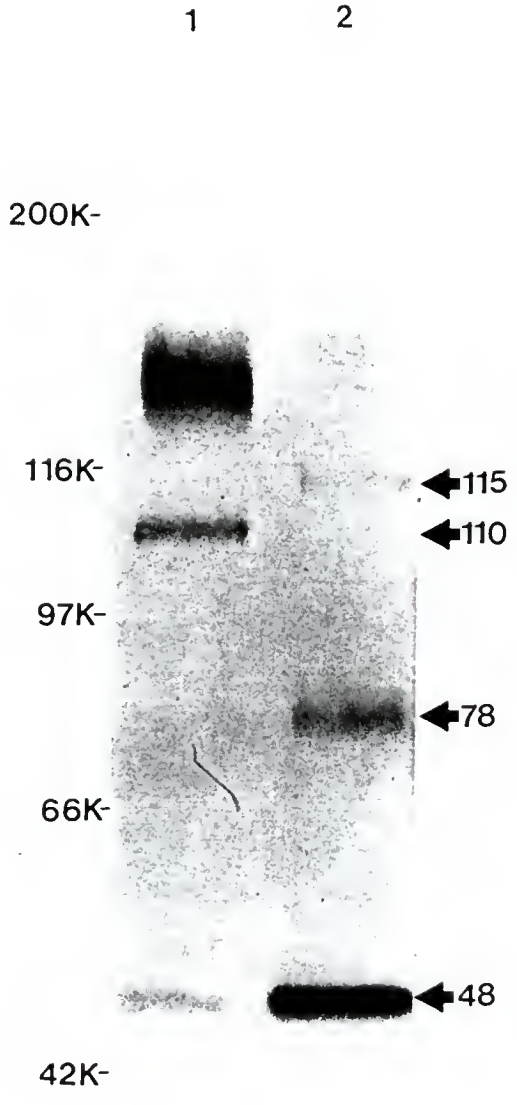


Figure 6.6 - SDS-PAGE analysis of $^{35}$S-methionine labeled AHV-1 110 kDa protein (lane 1) and 115 kDa protein (lane 2) excised from a non-reducing gel, electroeluted and subsequently run on a 9% polyacrylamide gel under reducing conditions. Numbers to right indicate molecular weights of proteins (x1000).

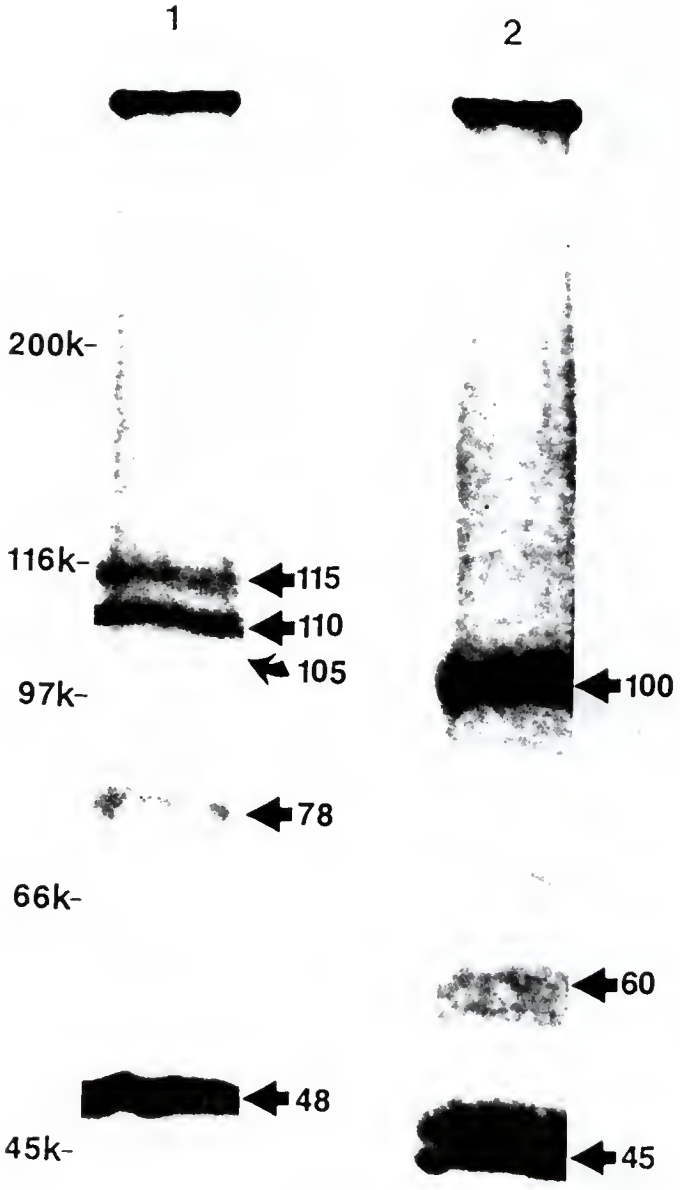


Figure 6.7 - SDS-PAGE analysis of $^{35}$S-methionine labeled AHV-1 proteins immunoprecipitated by monoclonal antibody 12B5 mock digested (lane 1) or digested (lane 2) with N-glycanase. Numbers to left indicate proteins immunoprecipitated from AHV-1-infected cells and their apparent molecular weights (x1000).

N-Glycanase digestion

Digestion of the immunoprecipitated 115 kDa complex with N-glycanase under reducing conditions resulted in a loss of the 115 kDa, 110 kDa and 105 kDa proteins, and the appearance of a new protein at approximately 100 kDa. The 78 kDa and 48 kDa proteins were not seen in the digested sample, but new proteins of 46 kDa and 60 kDa became apparent (Figure 6.7).

## Discussion

Pulse-chase experiments indicated that the first protein in the 115 kDa complex to be made was the 110 kDa molecule. We also knew from previous experiments that this molecule was glycosylated (Figure 2.5). This was consistent with the 110 kDa protein being the primary translation product to which N-linked sugars were added. The next protein to appear in the pulse-chase experiments was the 105 kDa protein. This protein was seen in small amounts under both reducing and non-reducing conditions, and is considered to be an intermediate form that possibly has undergone trimming of sugars. The 115 kDa molecule first appeared in the 30 minute chase period, and label continued to accumulate in this protein for the next 24 hours if samples were analyzed under non-reducing conditions. When the pulse-chase samples were run under reducing conditions, most of the 115 kDa molecule was replaced with a 78 kDa and a 48 kDa molecule. This provided evidence to suggest that the 115 kDa molecule was a heterodimer comprised of a 78 kDa and a 48 kDa molecule held together by disulfide bonds. Since the 115 kDa molecule appeared last in the pulse-chase experiment, we hypothesized that the 115 kDa molecule was

the mature form of the protein. Further support for this assumption was provided by the fact that when lysate of $^{125}I$ labeled virions were immunoprecipitated with monoclonal antibody 12B5 and run under non-reducing conditions, only one protein of 115 kDa was seen. When this same precipitated molecule was analyzed under reducing conditions, it was replaced with two proteins, one at 78 kDa and one at 48 kDa. A very small amount of the 115 kDa molecule was visible in auto-radiographs of gels run under reducing conditions. We hypothesized that this may represent a transient uncleaved form of gp115.

Since relative mobilities of proteins were slightly different when they were electrophoresed under reducing rather than non-reducing conditions, an experiment was undertaken to be certain that the 115kDa molecule represented the heterodimer. The 115 kDa and the 110 kDa proteins were excised from preparative gels run under non-reducing conditions, and subjected to reducing conditions before being separated by SDS-PAGE. The 115 kDa molecule was almost completely degraded into a 48 kDa and a 78 kDa protein. The 110 kDa molecule remained largely intact with a small amount of protein visible at 48 kDa. A new species, heavily labeled appeared at approximately 160 kDa. This probably represents aggregation of protein as a result of oxidation of cysteine residues during electroelution, as only a very small region corresponding to a molecular weight of 110 kDa was excised from the preparative gel for electroelution. The small amount of proteins at 48 kDa that could be seen when the 110 kDa molecule was

reduced with 2-mercaptoethanol we believe might result from breakdown of the 110 kDa protein at the cleavage site.

When the 115 kDa protein complex was immunoprecipitated with monoclonal antibody 12B5 and digested with N-glycanase under reducing conditions, the 115 kDa, 110 kDa and the 105 kDa molecules were replaced by a molecule of approximately 100 kDa. The 78 kDa and 48 kDa molecules were also replaced by molecules of approximately 60 kDa and 46 kDa. This would suggest that the 115 kDa, 110 kDa, and the 105 kDa molecules all have the same protein backbone and that processing probably does not involve addition of 0-linked sugars. These data also suggest that the 2 new molecules of 60 kDa and 46 kDa are the digestion products of the 78 kDa and 48 kDa proteins. After consideration of these data we propose the following biosynthetic pathway for the gp115 complex. The primary translation product gp110 is processed via a 105 kDa intermediate to a protein of 115 kDa. The 115 kDa molecule is cleaved into 2 proteins of 78 kDa and 48 kDa which are linked by disulfide bonds. This disulfide linked heterodimer is the mature form of the protein that is inserted into the virion envelope.

These studies, although not exhaustive, have provided a foundation for further evaluation of this apparently abundant protein that undoubtedly has some functional significance to the virus and may be useful as a reagent in diagnosis and possibly immunization. This information will prove invaluable in pursuit of these goals.

# CHAPTER 7

## SUMMARY

An appreciation of the biological activity of herpesviruses is dependent upon the understanding of the components, such as membrane proteins, that are important to the process of infection. We have been interested in examining the membrane proteins of Alcelaphine herpesvirus 1 with several long term goals in mind.

First, the virus of malignant catarrhal fever is a significant pathogen in cattle and results in a devastating disease. Studies of the roles that membrane proteins play in the initiation of immunity may in the future help to elucidate elements useful in the preparation of subunit vaccines. We have produced one monoclonal antibody 12B5 that recognizes a complex of membrane glycoproteins that we have designated gp115. The monoclonal antibody 12B5 is capable of neutralizing virus. This result suggests that gp115, which is expressed on the surface of the virion, warrants further evaluation as a potential protective immunogen. With the potential importance of this protein in mind, its processing was more thoroughly examined.

Second, we sought to help solve a problem that exists in veterinary medicine. Prompt identification of animals that may serve as carriers of AHV-1, and related viruses, is essential to effective control and containment of malignant catarrhal fever. Our first intention was to identify a protein unique to AHV-1 that was not

shared with the other herpesviruses infecting cattle. Our hypothesis was that if we could identify such a protein, we could use antibodies reactive with this protein as reagents in a diagnostic competitive ELISA test. No single protein could be conclusively identified as unique, therefore a different approach was taken. Monoclonal antibody 12B5 was confirmed to recognize an epitope unique to AHV-1, and animals known to have been infected with AHV-1 possessed antibodies recognizing this same epitope. The antibody was therefore shown to be a suitable reagent for use in a competitive ELISA assay. Results indicate that an assay of this type may prove to be useful in the diagnosis of not only AHV-1, but also possibly the sheep associated form of malignant catarrhal fever. One strategy for possible improvement of the specificity of this assay might include the use of portions of gp115 as antigen. Large amounts of gp115 could be obtained by cloning the appropriate gene into plasmid expression vectors. Since monoclonal antibody 12B5 recognized not one, but a complex of proteins, the precursor/product relationships of these molecules was further elucidated. Knowledge we obtained regarding the relationships between these molecules will be useful in designing cloning and selection stategies for genes encoding the protein complex.

Third, we are intrigued by the pathological similarities between AHV-1 and the human diseases associated with Epstein-Barr virus. Alcelaphine herpesvirus 1 might in the future represent an interesting animal model for the study of human gammaherpesviruses. This research goal was not specifically addressed in this dissertation, but

the identification and characterization of the virus membrane proteins that was accomplished will provide a useful foundation for the study of the role of membrane proteins in gammaherpesvirus-induced disease.

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## BIOGRAPHICAL SKETCH

The candidate, Steven Wade Adams, graduated from Grace Davis High School in Modesto, California. He earned a Bachelor of Science degree majoring in biological sciences at the University of California at Davis in 1980. In 1984 he completed a combined program in the School of Veterinary Medicine at the University of California at Davis. He was then conferred with the degrees Doctor of Veterinary Medicine and Master of Preventive Veterinary Medicine. He began graduate studies at the University of Florida in 1985 in the Department of Pathology and became a Ph.D. candidate in 1987. While pursuing his graduate studies, he also completed post-DVM training in comparative medicine in the JHMHC Animal Resources Department. He plans to pursue a career in academic laboratory animal medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May 1989

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